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PHYSIOLOGICAL REVIEWS

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PHYSIOLOGICAL REVIEWS

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THE REGULATION OF THE RESPIRATORY MOVEMENTS BY PERIPHERAL CHEMO-RECEPTORS

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1. The regulation of the respiratory movements by chemical influences on the respiratory centers has been demonstrated repeatedly since the classical observations of Rosenthal (1880) on the respiratory effects of oxygen-lack; of Pflüger (1868) on the influence of metabolites accumulated in the blood; and of Hermann (1870) on the action of carbon dioxide and the variations in excitability of the centers which depend upon the oxygen tension of the blood. Of particular importance are the experiments of Fredericq (1890), using for the first time the method of crossed circulation, and the more recent work of Haldane and Priestley (1905), Haldane and Poulton (1909), Henderson (1908–38), Winterstein (1911), Hasselbalch (1912), Hooker, Wilson and Connett (1917), Gesell (1925), Dautrebande (1930), Winterstein and Frühling (1934), and many others. The increase in the concentration of carbon dioxide in the alveolar air, in the blood and in the cells themselves which form these centers stimulates the pulmonary ventilation.

This action of carbon dioxide, or of oxygen-lack, on the various types of respiration in air or in water is found in the most diverse species of animals. It represents a universal biological phenomenon. It is evident in mammals, birds, amphibia and reptiles. Westerlund (1906), Olthoff (1936) and Powers and Clark (1942) observed it in fishes; Fox and Johnson (1934), Peters (1938) and Lindroth (1938) in crustacea; Winterstein (1925) in certain cephalopods, etc. It is interesting to point out that in aquatic respiration a low oxygen tension is more effective than the concentration of carbon dioxide in increasing the respiration (Meyer, 1935; Heerdt and Krijgsman, 1939) an effect which is not surprising considering the physical conditions of the medium. These important questions have been examined by Krogh (1941).

The control of pulmonary or branchial ventilation occurs through the action of the centers which are affected by the gas content of the internal environment in relation to the composition of the external environment. But the possibility of peripheral chemo-receptors which may evoke reflexes adequate for the respiratory needs must also be considered.

"For various reasons," I wrote in 1918, "we consider that in *addition to the central chemical influences* there must be a peripheral regulatory factor acting through receptors which are also sensitive to chemical changes. Just as the vagus transmits afferent impulses in response to mechanical stimuli at the level of the lung which have reflex effects, so there must be pheripheral receptors which are excited by variations in the concentration of carbon dioxide."

This hypothesis has classical antecedents. Donders (1853), Berns (1870) and Traube (1871) thought that the increase of carbon dioxide in the air contained in the lungs must be concerned in the regulation of the respiratory movements.

R.L.

Nevertheless, because of the lack of sufficient experimental proof, because of the study of the mechanical reflexes begun by Hering and Breuer (1868) and continued by Gad (1880), Zuntz and Geppert (1888), Head (1889) and many others, and because of the very important work of Haldane and his school and of those previously mentioned on the effect of the gas content of the blood on the functions of the respiratory centers which regulate the movements, no attention was paid to the peripheral chemical control of the respiration.

Starling stated in his *Principles of Human Physiology* (1915): "If . . . we succeed in altering the tensions of the two gases (oxygen and carbon dioxide) in the alveolar air we may assume that the tensions of the gases in the arterial blood leaving the lungs are altered in the same ratio." Through the action of chemoreceptors in the terminal portions of the respiratory apparatus, the composition of the alveolar air and therefore of the gas content of the blood is adjusted with the result that the ventilation would always be what the circumstances require. Only high concentrations of carbon dioxide in the inspired air, which increase the ventilation enormously, are able to raise the concentration of carbon dioxide in the alveolar air appreciably. The great speed of the responses, and the exactitude with which compensation is produced, lead one to suppose that there are reflexes evoked by chemical excitation from the respiratory apparatus which would operate previous to any humoral influence.

The experiments of Haldane (1922) show that if air with increasing quantities of carbon dioxide is breathed, it is possible for the ventilation rate to be doubled with practically no change in the composition of the alveolar air. Campbell, Douglas and Hobson (1914) observed that an increase of 2.5 mm. Hg in the carbon dioxide tension is sufficient to bring the ventilation to 10 liters per minute. These observations have been confirmed repeatedly (Campbell, Douglas, Haldane and Hobson, 1913; Douglas and Harvard, 1932; Barcroft and Margaria, 1931).

Nor, on the other hand, does rarefaction of the air change the gas content of the arterial blood appreciably, as has been known since the work of Fränkel and Geppert (1883): at a pressure of 410 mm. Hg there is no change in the gas content of the blood; nevertheless the respiratory dynamics have been modified.

The results obtained by Scott (1908) furnish very interesting data: without the vagi, the control of the respiration is uncertain, the reactions are slower and last longer after a carbon dioxide mixture has been breathed, and in general the adjustment of the respiration to physiological requirements is inexact. If the respiratory movements depended solely upon the humoral influence on the centers, and the vagus were limited to the rôle of a sensory nerve for mechanical receptors, there would not be such marked differences in the respiratory reactions to inspired carbon dioxide when the vagus is intact and when it has been cut. Our experiments designed to clarify this problem have been numerous and of various types.

At first we studied (1918) the behavior of the respiration in vagotomized dogs exposed to the same concentrations of carbon dioxide in air, before and after the operation. Observations were made immediately after the section of the vagi or some hours later. The differences with respect to the normal were always

noteworthy in that they showed lack of precision and delay in motor reactions when the vagal innervation was absent. Our conclusions were: 1. The constancy of the proportion of CO_2 in the alveolar air contradicts the hypothesis that the respiratory stimulation is exclusively of central origin evoked by the excess of CO_2 or lack of O_2 in the blood. 2. The lung and the bronchial ramifications are sensitive to different chemical stimuli, which can evoke reflexes. 3. In addition to the well known action on the respiratory centers, there is exerted a parallel or perhaps a previous peripheral influence due to the excitation of end-organs which are sensitive to stimuli of chemical nature by the CO_2 contained in the inspired air.

The following year in collaboration with Bellido (1919–21) and as a more evident demonstration we devised a crossed-circulation technique, otherwise known as the "dog with two heads." The central stumps of the carotids and jugulars of the donor dog "A" are joined to the cephalic stumps of the corresponding vessels of "B," the dog in which the experiment is performed. The vertebral arteries of the latter dog are also ligated, and the animals are chosen so that "A" is sufficiently larger than "B" and its carotid pressure higher, thus preventing the head of the recipient from receiving its own blood through the intravertebral plexuses. C. Heymans and Ladon in 1925 and J. F. and C. Heymans in 1926 proposed a definitive method similar to ours except that they cut off the head of "B" (method of the isolated head) and record respiratory movements of the larynx and floor of the mouth. With Puche (1930) we studied various ways of registering these movements with the purpose of improving the recording. Isolation of the head assures that only the blood of the donor dog reaches it, because it is connected to the trunk only by the vagi.

When the respiratory centers of "B" are perfused by either of the two procedures with the blood of "A," which is breathing normally or in some of the experiments is given artificial respiration, and "B" or its trunk which has been separated from the head is made to breathe air with CO_2, an increase in the frequency and depth of respiration is observed.

With Puche and Raventós (1930) we used also the technique of the isolated head. As in our first experiments of 1919 and succeeding years, if after decapitating "B," leaving the head which is connected with the trunk only by way of the vagi, we caused the trunk to breathe air with CO_2, the head responded with an increased intensity of the respiratory movements, despite the fact that it was being perfused with normal arterial blood from the donor dog "A."

The researches of J. F. and C. Heymans (1926–28) showed that the head, besides responding to mechanical stimulation of the pulmonary vagal endings (Hering-Breuer reflex), also responds to peripheral chemical stimuli. A reflex apnea is produced by over-ventilation as described by Hering and Breuer (1868) and afterwards studied by Baglioni (1903), Foa (1909–11), Githens and Meltzer (1914), Joseph (1922), Puche (1923), Meek (1923) and others. This apnea has been generally attributed to over-distention of the lungs, but Heymans and Heymans state that the major factor in producing it is the peripheral sensitivity to the concentration of CO_2 in the blood. Such a sensitivity was suggested by

Luciani (1888) and Bordoni (1888). Anemia of the trunk with asphyxia of the tissues causes acceleration of breathing, thus confirming the possible peripheral origin of the dyspnea in addition to its central origin, a thesis which had been proposed earlier by Francois Franck (1890), Hoffmann (1900), Porter and Newburgh (1916–17) and Dunn (1920). Heymans and Heymans were able to demonstrate also the relation between the cardio-aortic pressure and the breath rhythm, and, a fact which interests us particularly, respiratory responses to changes in the composition of the inspired air and in the gas content of the blood circulating through the heart and great vessels.

Heymans, Bouckaert and Regniers (1933) write: "J. F. and C. Heymans observed that intense hyperventilation of the trunk of dog 'B' causes reflex apnea of the isolated head of the same animal; on the other hand, progressive asphyxia of the trunk by withholding artificial respiration *or the administration of air with carbon dioxide*, causes the reappearance of respiratory movements of the head followed by a progressive increase in their amplitude as the asphyxia becomes more severe." Heymans and Heymans wrote in 1927: "These experiments taken together show that the vagi contribute to the reflex regulation of the activity of the respiratory center according to the peripheral respiratory and circulatory conditions. The respiratory center can be excited in the same way by a peripheral state of asphyxia or anoxemia as by a central state." Having demonstrated the existence of respiratory reflexes evoked by chemical stimuli Heymans and Heymans (1927) then set out to discover the peripheral origin of the respiratory tone and of the vagal influence on the respiration. For this purpose they performed various series of experiments demonstrating that the cardio-aortic intraceptive zone is the place of origin not only of circulatory reflexes but of respiratory reflexes as well and that the stimuli can be chemical as well as mechanical. "The intrapulmonary or humoral accumulation of carbon dioxide in the trunk constitutes the vagal reflex stimulus of the respiratory center of the isolated head."

From their experiments Heymans and Heymans concluded that the respiratory reflexogenic zones which are affected by chemical stimuli are found in the central organs of the circulation, the heart and the aorta, and that the lungs have no specific excitability to carbon dioxide. In our judgment this last proposition has not been confirmed. In the first place it should be remembered that it is more or less asphyctic blood circulating through the lungs which is used as a stimulus and *not the inspired air*, which would affect the whole respiratory tree, from the nose to the pulmonary alveoli. Thus it is rather the functions of a reflexogenic zone in the circulatory apparatus which are being investigated—the sensory innervation of the pulmonary vessels *stimulated by changes in the composition of the blood*—and not whether the respiratory pathways respond to the composition of the air. Again, the important operative intervention, extirpation of the heart and great vessels, may suffice to put the animal in an unphysiological state; and one must reckon also with the possibility that many sensory fibres of the pulmonary plexuses which pass to the vagi may be damaged by the same operation. All of this may damage or alter the vagal sensitivity and cause the vagotonic type of respira-

tion observed and the scarcely appreciable reaction to the circulation of the hypercapnic blood. It is invalid to argue that the Hering-Breuer reflex, which is known to be very rough, is still present. Anrep and Samaan (1933) have shown in denervation experiments that a very few remaining fibres are sufficient to cause this reflex to persist.

After the demonstration by Heymans and Heymans of the existence of circulatory reflexogenic zones with chemo-receptors we decided to return to the subject of pulmonary sensitivity. In new publications with Puche (1930) and with Raventós (1931–33) we employed the technique of the isolated head as usual, and in order to exclude all circulatory influence we bled the trunk "B" completely and rapidly by cutting the abdominal aorta until the heart stopped. Thus all circulatory factors both mechanical and chemical are suppressed since the blood no longer circulated. It is certain that with this bleeding the sensitivity of the lungs is diminished; but this fact, which furnishes a valid objection to negative interpretations, is favorable the other way around; in the normal state the reflex would logically be more intense and more effective.

With the addition of CO_2 in varying proportions to the air going into the trunk, while identical mechanical conditions of respiration are maintained, it is shown that even in such unfavorable circumstances the inhalation of CO_2 intensifies the movements of the isolated head which are already increased as a result of hemorrhage and subsequent asphyxia of the tissues.

We have tested also the response to the inhalation of irritant gases, hydrogen chloride and ammonia, and confirmed the observations made with Bellido (1919).

Heymans has (1929–33) raised the objection to the conclusions from these experiments that in the production of the reflexes observed it is not a specific sensitivity which is acting, but the general sensitivity to irritant agents. "The results obtained by Pi-Suñer and Bellido," state Cordier and Heymans (1935) "are certainly due to the fact that these authors have administered by inhalation air with concentrations of CO_2 which pass beyond physiological limits and even beyond the pathological. It is a question of phenomena of pharmacological order." It is difficult to know where a specific chemical excitation ends and where an irritant, nociceptive excitation of chemical origin begins, and where is located the boundary which separates physiology from pharmacology. HCl and NH_4OH for example do not increase the depth and rate of respiration like CO_2, but they inhibit it or provoke abnormal reactions: cough, spasm, etc. Barcroft and Margaria (1932) observed that high concentrations of CO_2 cause a characteristic type of respiration, which is not a simple increase but may be faster or slower depending upon the previous rate. After section of the vagi the breathing of high concentrations of CO_2 in air is usually followed by completely abnormal symptoms analogous to those observed by Lumsden (1923) upon sectioning the medulla at various levels, and similar to those which Taylor (1930) observed after the administration of cyanide: apneusis, gasps and finally paralysis. Some of our positive results have been obtained with mixtures of air and CO_2 which are closer to the normal and in which it cannot be said that carbon dioxide acts as an irritant gas.

The negative results of Partridge (1933), who attempted to record vagal action currents using inspired CO_2 as a stimulus, have also been cited in opposition to the theory of pulmonary chemical sensitivity. This is a problem which we began to study with Bellido in 1921. The electrovagograms which we obtained then showed certain differences when the animal breathed carbon dioxide, but were not conclusive. Our technique at that time was deficient. Adrian (1933) states that concentrations of CO_2 in air higher than ten per cent cause a modification, a slight diminution in the intensity of the impulses passing in the vagus when the lung is distended, but that these effects are small and within the limits of possible experimental error. Bullring and Whitteridge (1943–44) say that an increase in vagal electric discharge—single fibre preparation—occurs as soon as a volatile anesthetic—ethyl chloride, chloroform, ether, divinyl ether, trichlorethylene, etc.—reaches the lung, whether it is administered by inhalation or intravenously. But it is not possible to state that the respiration of air with carbon dioxide in greater concentration than normal is accompanied by definite signs in the electrovagogram.

In a later series of experiments (Pi-Suñer and Raventós, 1931) we have returned to the question, making the conditions of observation more rigorous even at the cost of simplicity. Using the method of the isolated head, we placed a cannula in the pulmonary artery and another in the left auricle of the trunk of "B." All of the rest of the heart and the thoracic aorta were removed, care being taken to avoid damage insofar as possible to the nerve fibres which form the plexuses at the roots of the lungs and their vagal continuation. Defibrinated, oxygenated, blood was perfused through the lungs by means of a Dale Schuster pump. Thus only the lungs and head of dog "B" remained alive, connected by the vagi which had been cut below the heart. After this procedure it is still possible to obtain respiratory responses to the inhalation of air with CO_2. It is evident that such reflexes cannot originate in the heart and aorta which are no longer present, and that they are due to the influence of the inspired carbon dioxide upon lungs which are perfused with blood of constant gas content.

Another series of experiments recently completed (1938–42) consisted in denervating the heart and great vessels while leaving the pulmonary innervation intact insofar as possible. Again we used the technique of the isolated head perfused with blood from a donor dog "A." Relying upon the anatomical findings of Lim (1893), Cannon, Lewis and Britton (1926) and Barry (1935), we established a technique of intrathoracic denervation of the heart similar to that described by Anrep and Samaan (1933) and Anrep, Pascual and Rossler (1932).

With this procedure the breathing of air with CO_2 by the trunk gives rise to changes in the respiratory movements of the head. Now the responses to chemical stimuli cannot arise in the heart and aorta which have been denervated, nor from the carotid sinus which, as in all the experiments with the isolated head, remains in the head and is perfused by blood from the donor dog.

It is important to mention the work of Dirken and Van Dishoeck (1937) who also demonstrated the sensitivity of the lung to changes in the concentration of CO_2 in the alveolar air and concluded that the nerve endings present in the lungs

are sensitive to changes in the concentration of CO_2 such as are found normally in alveolar air, i.e., 5 to 6 per cent.

Dirken and Van Dishoeck consider furthermore that the sensitivity of the pulmonary receptors is limited to CO_2 because they did not note any difference in the reflex respiratory responses, when the proportions of oxygen and nitrogen were varied in the breathed gas mixtures. Bilateral section of the vagi abolished the reflex effects of changes of CO_2 in the air.

After confirming the sensitivity to CO_2 of pulmonary and bronchial chemo-receptors, the authors reach a paradoxical conclusion: these receptors are not the instruments of the normal regulation of the respiratory movements. They observed in some experiments that as the frequency of the inspirations increased their amplitude was reduced, and they inferred from this fact that the ventilation was not changed. They doubt that the phenomena observed depend upon a direct sensitivity to carbon dioxide on the part of the pulmonary endings, attributing them rather to changes in the excitability of the receptors for their natural physiological stimulus: the collapse and the expansion of the lung evoking reflexes through mechanical stimuli. However the fundamental fact is the existence of endings which are sensitive to changes in the concentration of CO_2 in the alveolar and bronchial air and which influence the movements of respiration.

Recently Hammouda, Samaan and Wilson (1942–43) reaffirmed once again that the fibres in the vagi which conduct the afferent impulses of the reflexes of inflation and deflation are of pulmonary origin, and Bagoury and Samaan (1941) have studied respiratory reflexes by injecting ketone-bodies into the pulmonary circulation. The authors prevented any possible central action of the blood; the vagal excitation arose from sensory endings of the lungs. These pulmonary endings would therefore be sensitive both to mechanical and to chemical stimuli.

Eppinger, Papp and Schwartz (1924) infer the mediation of pulmonary chemical sensitivity in studying the mechanism of cardiac asthma; Dunn (1920) and Binger and Moore (1927) consider also local pulmonary influences when they attempt to interpret the hyperpnea of pulmonary embolism, and Churchill and Cope (1929) do likewise in considering the dyspnea observed in pulmonary edema and which they attribute to the excitation of sensory endings in the lung.

Another important aspect is the debated intervention of the lung in the maintenance of vagal tone. Heymans and Heymans observe a disappearance of the respiratory tone, maintained by the vagus, when the stimuli arising in the heart and aorta are no longer present and only those arising in the lungs remain. Heymans and Heymans conclude that the lungs are not the site of origin of the centripetal vagal impulses which maintain the physiological respiratory vagal tone and the reflex excitation and inhibition of the respiratory center in relation to respiratory and circulatory mechanisms.

Our observations demonstrated that after bleeding the trunk and therefore excluding all circulatory influence either mechanical or chemical, the normal rhythm of the respiratory movements of the head continues. *It is only after section of the vagi that respiration of the post-vagotonic type appears.* This suggests

that at least the sensory innervation of the lung assists in maintaining the vagal tone.

The latter proposition is confirmed by the investigations of Anrep and Samaan (1933). These authors cite the work of Pavlov (1895–96) and his pupils and collaborators, Cachovsky (1899) and Cheshcov (1902), studying the respiratory movements in vagotomized dogs surviving up to nineteen months after operation. Just as in the acute experiments, the rate is reduced to 4–8 per minute immediately after the section of the vagi, speeding up in the second or third week, and slowing down again to the same original bardypneic rate which is maintained until the death of the animal. Pavlov attributed the initial slowing to the cutting of sensory fibres of pulmonary origin and the subsequent acceleration to a process of irritation in the same fibres with scarring of the central stump of the cut nerve. When the process of cicatrization is completed the rhythm characteristic of the absence of vagal afferent impulses is re-established. Sharpey-Schafer (1932) states that the respiratory slowing is due to the increased resistance to the passage of air through the respiratory pathways owing to paralysis of the laryngeal nerves. It has not been difficult to demonstrate the error of this opinion. Heymans and Heymans (1927) assert that the post-vagotonic rhythm is due to the lack of cardio-aortic control. The "respiratory tone" is of cardio-aortic origin and disappears when the vagal fibres arising in that region are cut. "No direct evidence in support of this view is, however, provided," write Anrep and Samaan, who have shown that section of the vagal fibres at the level of the root of the lung does not alter the cardio-aortic innervation and that the branches which leave below this point go to the lung, with the exception of a few which pass to the esophagus. Taking into account these anatomical findings, Anrep and Samaan (1933) denervated the heart and aorta by means of intrathoracic dissection without producing the respiratory rhythm typical of vagal section. The section of one of the vagi and cardio-aortic denervation on the opposite side did not cause vagotonic respiration either. Later section of the remaining vagus in the neck caused this type of respiration to appear at once. Moreover, they sectioned the vagus below or at the level where the pulmonary branches leave the main trunk and compared the different results in the two cases. With the low section no differences in rate were observed; with the high section the characteristic slow respiration appeared; it switched from 24 to 7 respirations per minute. Subsequent bilateral vagotomy in the neck no longer modified this slow rate. This observation produces additional evidence against the cardio-aortic theory as well as against the laryngeal theory of Sharpey-Schafer.

Bouckaert and Heymans (1933) comment: "The reflex and tonic influences of the pulmonary vagus on the activity of the respiratory centre is a well-known and generally accepted fact (Hering and Breuer, 1868; Head, 1889; Haldane, 1922; J. F. and C. Heymans, 1926; C. Heymans, 1928; Hoffmann and Keller, 1929; Hess, 1931; Anrep and Samaan, 1933). It has been shown by J. F. and C. Heymans (1926) and C. Heymans (1928, 1929a, b) that the vago-depressor nerves in dogs are also the centripetal paths of respiratory reflexes in relation with the

cardio-aortic blood-pressure .... The normal arterial pressure in the left heart and aortic arch maintains a respiratory reflex tonus. Anrep and Samaan (1933) cannot find any evidence that cardio-aortic impulses invariably exert on the rate of the respiration a regulating influence which can be compared with the definite and constant dependence of the respiration rate on the pulmonary innervation.

Recently Sidney Harris (1945) has studied the behavior of the respiratory tonus in anoxia. During the reduction of oxygen content in inspired air to about 8 per cent the volume of the cat's chest at the end of expiration increased about three times the volume of a normal respiration. After vagotomy the anoxic increase in expiratory volume was about one-third as great as with the vagi intact. Crushing the nerves from the carotid chemo-receptors had little or no effect upon the reaction. An excess of the CO_2 in the oxygen deficient air did not prevent the increase in respiratory chest volume, but it did greatly increase the minute-volume of respiration. Gesell and Moyer (1934–35) and Green and Swanson (1938) had earlier reached similar conclusions.

Hermann, Jourdan and Vial (1934) suggest as a result of their experimental observations that the tone of the pulmonary vagus is continuous, depressing the activity of the cardio-inhibitory center and eliciting circulatory pressor reflexes which are antagonistic to the depressor reflexes arising in the cardio-aortic sensory area and in the sensory area of the carotid sinus. These conclusions, perhaps too schematic, are nevertheless of interest because they are based on experiments which demonstrate once again the rôle of the pulmonary vagus in controlling the respiratory tonus.

The existence of sensory endings in the respiratory apparatus should now be considered. Schumacher (1902), Hudovernig (1907), Tello (1924), Perman (1924), Gaylor (1934), demonstrated these receptors in the alveolar duct and the presence of fibres of pulmonary origin in the vagus nerve. Larsell (1921–39), Larsell and Burget (1924) and Sunder-Plassmann (1933) describe sensory nerve endings in the mucosa of the bronchi and trachea which are probably sensitive to mechanical stimuli. According to Larsell (1939), receptors of a different morphological type are found in the depths of the respiratory tree and it may be inferred that they are chemo-receptors because of their location directly in the air passage.

The fibres proceeding from all these endings ascend with the vagus, but Beccari (1934) believes that some afferent respiratory fibres also pass with the sympathetic, a fact in accord with the scheme of double sensory innervation of the vegetative organs through the sympathetic and parasympathetic outlined by Pi-Suñer and Puche (1928–30). Brookhart and Steffensen (1936) observe respiratory effects when the stellated ganglion is ablated, but they do not conclude that the afferent fibres implicated in the Hering and Breuer's reflex should necessarily pass through it.

Numerous facts, established by us and by other authors, demonstrate the chemical sensitivity of pulmonary origin. Heymans and Heymans have shown that the cardio-aortic areas are sensitive to chemical stimuli, and they think that the latter sensitivity is linked to the circulatory apparatus and excludes the

pulmonary chemical one. We consider, however, that no incompatibility exists; that, on the contrary, the effects of peripheral excitation of separate origin support each other functionally.

II. It is a well established fact that different sensory regions operate synergistically in the control of distinct vegetative functions. In the case of the respiratory and circulatory reflexes the sensory regions are various and do not hinder the function of one another but rather coordinate their regulatory influences.

Since the early observations of Pagano (1900) and Siciliano (1900) and the description by Hering (1927–32) of the functions of the carotid sinus, a number of authors have devoted themselves to this very important reflexogenic zone. The sinus is concerned not only with the regulation of circulatory dynamics under the influence of changes in the blood pressure, but its action extends also to various functions; among these is the respiratory.

"Many clinicians," write Heymans, Bouckaert and Regniers (1933), "have called attention for some time to the fact that carotid compression elicits changes in respiration." Tschermack (1866), Quincke (1875), Sollmann and Brown (1912), Danielopolu, Asland, Marcou, Proca and Manescu (1927), Danielopolu, Manescu and Proca (1928), Wenckebach and Winterberg (1927) proved that traction on the cephalic stump of the recently cut carotid awakes reflexes through mechanical excitation of the sinus. Similar findings have since been demonstrated repeatedly: Gollwitzer-Meyer and Schulte (1931), Schmidt (1932), Winder, Winder and Gesell (1933), Gemmill and Reeves (1933).

Moisejeff (1927) demonstrated that such effects originate in the sinus. Upon ligating the vessels which arise from the bifurcation of the carotid and perfusing the sinus he found inhibition of the respiratory movements when the pressure of the perfusing fluid increased. C. Heymans (1929) and Heymans and Bouckaert (1929–30) have confirmed these results. Afferent nerve impulses arise in the carotid sinus as a result of mechanical excitation and intervene in the process of regulation of the respiratory dynamics just as in the case of the circulatory. Houssay and Orias (1934) have studied the effects of excitation of the sinus on the contraction of the smooth muscle of the bronchioles and have observed inconstant reflex effects.

But not only do the mechanical conditions of the circulation influence the respiration through carotid and aortic body reflexes. *The composition of the blood is equally effective*, particularly the content of CO_2 and oxygen.

Heymans, Bouckaert and Dautrebande (1930–31–32) using the previously described technique of Moisejeff, discovered that acapnia of the blood produces reflex inhibition of the respiratory center while blood with an excess of CO_2 stimulates the center. According to Heymans, Bouckaert and Regniers (1933) the most effective chemical stimuli for the carotid body are hypercapnia, anoxemia and increased concentration of hydrogen ions. There immediately appeared numerous confirmations: Owen and Gesell (1931), Schmidt (1932), Selladurai and Wright (1932), Bernthal (1934), Heymans, Bouckaert and Samaan (1935), Gayet, Bennati and Quivy (1935), Samaan and Stella (1935), Zottermann (1935), etc. Slight changes in the composition of the blood passing through the

carotid sinus give rise to vigorous circulatory and respiratory responses. Hill and Flack (1908), Piras (1922), Frey (1923) and Hess (1931) did not believe that the respiration could be influenced normally in reflex fashion by a greater or lesser concentration of CO_2 in the blood. Hering (1932) and Mies (1932) discuss the chemical sensitivity of the carotid body; Gollwitzer-Meyer and Schulte (1931) and Gollwitzer-Meyer (1934) the specificity of the sensitivity to CO_2. Such a mass of confirmatory results was so gathered together that there is no possible doubt.

Anatomical investigation has also been here of great value. De Castro (1926-28) began the studies by describing a complex system of receptors in the carotid sinus. He explored immediately the carotid body or glomus at the bifurcation of the carotids in anatomical continuity with the sinus. It is a small organ which has the appearance of an endocrine gland, possesses certainly an intense metabolism and among its cells is distributed a profuse net of nerve endings. Sunder-Plassmann (1930) furnishes interesting data and Heymans, Bouckaert and Regniers (1933) give in their turn a description of the region. Ask-Upmark (1935) has studied the comparative anatomy of the sinus in twenty-seven species of animals. Nonidez (1935–36) and Boyd (1937) have found glomerular tissue in other vaso-sensory regions: principally in the aorta (Penitschka, 1931)—"Glomus aorticum"—and in the vicinity of the pulmonary artery—"glomus pulmonale." There are other analogous cellular formations, e.g., the coccygeal body. And perhaps one might refer to this group of small organs the groups of cells attached to or near the peripheral vessels described by Goormaghtigh (1935) which he considers to belong to the neuro-vegetative system. The glomera are probably a part of the chromaffin system even though their cells do not usually show the characteristic protoplasmic granules which are stained by chromates. The glomera seem to respond especially to chemical stimuli, above all those which are found in asphyxia: anoxemia, hypercapnia, acidosis.

In 1931 Heymans, Bouckaert and Dautrebande observed that ligature of the nerve fibres arising in the carotid sinus prevents reflex adaptation to changes in the pressure of the blood contained in the sinus but does not abolish the reflexes elicited by chemical stimuli. And reciprocally according to Heymans and Bouckaert (1932) it is possible to inactivate the chemo-receptors by producing emboli with lycopodium powder in the vessels of the glomus while preserving the sensitivity to pressure. Gollwitzer-Meyer and Schulte (1931) and Gollwitzer-Meyer (1934) observed that lobeline exerts its action, which is identical with that of anoxemia, only when it reaches the carotid body. Danielopolu, Asland and Marcou (1933) also separate the location of presso-receptors of the sinus and chemo-receptors which are in the carotid bodies. Camus, Bernard and Merklen (1934) brought new confirmation by cutting the fibres from the presso-receptors, and Comroe and Schmidt (1938) conclude that the receptors for pressure are found particularly if not exclusively in the carotid sinus itself, while the chemo-receptors are at the origin of the occipital artery near the carotid body and probably in the carotid body itself.

The study of the electrical variations produced in the nerve of Hering due to

the functional state of the carotid sinus has given data which demonstrate also the topographical separation of the receptors. Bronk and Stella (1932) observed that action currents in the nerve result not only from distention of the sinus by increase in the blood pressure, but can be produced also by other kinds of stimulation. Heymans and Rijlant (1933) showed the presence of impulses in the same nerve which bore no relation to the pressure in the sinus and were dependent on the state of ventilation of the animal: when the ventilation is poor the discharges are more intense; the excitation which sets up the impulses is not mechanical but chemical depending upon the gas content of the blood. Bogue and Stella (1934–35), Zottermann (1935), Euler, Liljestrand and Zottermann (1939) confirm these observations in experiments on cats and suppose that the starting point of the impulses is the carotid body. Bronk and Stella (1934) confirm the independence of the chemo-receptors and the presso-receptors. Samaan and Stella (1935) have studied the influence of changes in the composition of the blood on the action currents in the nerve from the carotid body and have shown that these currents cease when the tension of carbon dioxide in the blood is reduced below 32–35 mm. Hg. The physiological tension of carbon dioxide in the arterial blood will maintain therefore a tonic excitation of the carotid chemo-receptors and through them of the respiratory centers as has been shown by Selladurai and Wright (1932) and by Witt, Katz and Kohn (1934). Euler, Liljestrand and Zottermann (1939) agree with the earlier conclusions but consider that the physiological tension of oxygen is equally as effective as that of carbon dioxide.

In the aorta the two types of receptors, mechanical and chemical, are likewise found separated from one another. Comroe (1939) has studied the aortic chemo-receptors and particularly the aortic body which is similar to the carotid body, and has established that the bodies are in both cases the point of origin of circulatory and respiratory responses to anoxia; in the dog the aortic receptors are said to elicit reflexes which are predominantly circulatory while the carotid body is the principal point of origin of respiratory reflexes. There are nevertheless individual differences, and in the cat the functional specialization does not seem to be so clear. Gellhorn and Lambert (1939) believe that the circulation is regulated by the presso-receptors while the respiration depends principally on the chemo-receptors.

III. With the demonstration of the chemical sensitivity of the various vascular zones, the most important of which are the carotid sinus and the aorta, it was logical to study the physiological significance of this sensitivity in comparison with that of the respiratory centers. "Central chemical control," writes Gesell (1939) "which had been accepted on faith, was not on the defensive. In the confusion, many adopted new faiths which carried them too far, for luck was with the majority. This they do with the same enthusiasm as their predecessors who thought that the respiratory regulation and also the circulatory was carried out exclusively by the state of the blood supplying the centers." Schmidt and Comroe (1941) state: "For many years the physico-chemical aspects of respiratory control were dominant, but during the past decade the reflex factor has grown

steadily in stature until, in our opinion, the time has come to reverse the traditional attitude".

In making a comparison between the central and the peripheral effect of the chemo-receptors Heymans considered that, at least in the case of anoxemia, the effect of excitation of the sinus takes precedence over that of the centers in the control of the movements of respiration. Heymans, Bouckaert and Dautrebande (1930) observed that as a result of denervation of the vaso-sensory zones, only a moderate acceleration of the rate of respiration and a slight increase in the blood pressure is produced when the dog breathes nitrogen without oxygen, instead of the response of violent hyperpnea and extreme hypertension by the normal animal.

A number of investigators have obtained similar results. Selladurai and Wright (1932), Witt, Katz and Kohn (1934), Euler and Liljestrand (1936), Euler (1938), and Gesell and Lapides (1938) observed that in general respiration is depressed after simple denervation of the carotid sinus. Schmidt (1932) and Gemmill and Reeves (1933), studying the immediate effects of denervation of the sinus, had shown that anoxia does not produce an increase in the pulmonary ventilation, but rather a decrease. Schmidt and Comroe (1940–41) and Moyer and Beecher (1942) agree in the statement that hypoxia increases the respiration of animals whose vaso-sensitive zones have been deafferented. Nevertheless these animals have gone through a long period of depression. Watt, Dumke and Comroe (1943) assert also that anoxia produces stimulation of the vaso-chemo-receptors while it leads primarily to depression of the centers. In experiments in which the denervation had been carried out previously, performed when the animal had recovered from the operation ("chronic" experiments), Gemmill, Geiling and Reeves (1934) observed a slight excitation produced by anoxia, but only when it was not excessive. Wright (1934) in "acute" experiments on anesthetized rabbits and later in chronic experiments confirms the respiratory depression caused by anoxia after carotid denervation. Smyth (1936–37) states that this depression is considerable and, moreover, anoxia reduced the intensity of the response to CO_2 in denervated animals. In the rabbit Stella (1935) does not find significant effects of denervation: the respiration may decrease in depth but increase in rate. Schmidt, Dumke and Dripps (1939–40) have blocked the sinus nerves with procaine and observed scarcely any change from the normal respiration.

Wright (1936) remarks that after denervation of the sinus and bilateral section of the vagi, oxygen-lack does not produce hyperpnea. After the sensory nerves of the carotid sinus and the aorta have been removed Wright finds that severe anoxemia in anesthetized cats instead of causing respiratory activation produces a reduction in the ventilation. The changes in the respiratory gases in the blood are greater in the operated animals than in the normal. He concludes that up to a certain point the vascular receptors in the carotid body and the cardio-aortic area protect the centers from chemical influences "in loco."

Schmidt (1932), Beyne, Gautrelet and Halpern (1933); Winder (1933);

Mulinos (1934); Henderson and Greenberg (1934); Wright (1934–36–38) Gayet, Bennati and Quivy (1935); Brewer (1937), Gesell and Moyer (1937); Smyth (1937); Comroe and Schmidt (1938); Gellhorn and Lambert (1939); etc., agree that anoxemia is the most effective stimulus for the vascular chemo-receptors, particularly of the carotid body.

Bernthal (1938) by perfusing the isolated carotid sinuses with blood of fixed pH and varying the concentration of oxygen, observed that 18 per cent oxygen causes at the most a moderate vaso-dilatation and hypopnea, while 15 per cent oxygen gives rise to vaso-constriction and hyperpnea an effect intensified when the proportion of oxygen is reduced to 12.8 per cent. A drop to an oxygen tension of 10 mm. Hg causes a marked increase in vaso-constriction and respiratory rate.

In spite of these facts, Cromer and Ivy (1931) noted that dogs from which the carotid sinuses had been removed aseptically, work in the treadmill without greater effort than normal dogs and do not show any respiratory disturbances. It should be noted however that in these dogs the cardio-aortic receptors remained intact. Decharneux (1934) also observed adequate respiratory responses afte denervation of the sinus. Dautrebande (1937) stated that dogs with complete deafferentation of the sinus and aorta respond to low atmospheric pressures in the same regular and adequate fashion as do normal dogs. Gesell (1939) cites cases of hyperpnea in response to anoxemia after denervation of the sinus.

It has long been known that the cyanides and sulfides inhibit oxidative processes in the organism. The mechanism of action of cyanides is well-known. Cyanide inhibits cellular oxidations by combining with cytochrome oxidase. Sulfides act in the same manner. A. Pí-Suñer and J. Pí-Suñer (1928–29) affirm that to the extent that the cyanides impede the processes of oxidation, in particular of glucose and fat, they evoke trophic reflexes which produce hyperglycemia and hyperlipemia.

Haggard and Henderson (1922) have studied the effects on the respiratory movements of sodium sulfide injected intravenously. Heymans and Heymans (1927) and Heymans, Bouckaert and Dautrebande (1930) showed, and it was quickly confirmed by Owen and Gesell (1931), that the injection of sulfide or cyanide into the carotid sinus causes very intense respiratory responses. Winder and Winder (1933) examined the action of the sulfides and called attention once more to the strong respiratory reflex effect elicited from the chemo-receptors of the carotid body. Denervation of the sinus modifies the response: doses which were previously extremely effective evoke, after denervation, a very different type of response of slight intensity. In all cases the reactions to stimulation of the sinus are more intense than those obtained by direct application to the centers. Winder, Winder and Gesell (1933) required quantities 25 to 75 times as great after denervation of the sinus to produce occasional respiratory effects. Winder's (1937) statement that monoiodo acetic acid acts upon the carotid body in a similar way as cyanides and sulfides is not in agreement with the general opinion that the first is an inhibitor of sulfhydryl enzymes while cyanide inhibits cytochrome oxidase.

These drugs also act upon the respiratory centers. Winder, Winder and Gesell (1933) applied cyanide directly to the fourth ventricle but noted that the respiratory movements were simulated only by larger doses than those required on the sinus; further increase in the doses caused difficulty of respiration which finally ceased. Injection of sulfides and cyanides into vertebral artery requires larger doses in order to alter the respiratory movements than are required with injection into the sinus.

Another factor which should be kept in mind in considering the action of cyanide or sulfide is the rôle of the vagus. According to Haggard and Henderson (1922) the respiratory effects in these cases are due to the action of the drugs on the vagal endings of the lung, in view of the differences in the responses depending on whether one is dealing with intact or with vagotomized animals.

Winder (1937), Bernthal and Weeks (1939) arrived at the conclusion that changes in pH in the blood and in the sensitive cells are a prime factor in normal carotid body stimulation, and Von Euler, Liljestrand and Zottermann (1939–41) confirm that intracellular acidity in the carotid body is increased during anoxia.

Dripps and Comroe (1944) divide the drugs that affect the sensibility of the carotid body in two groups: those that, like cyanides and sulfides, produce effects by inhibiting intracellular respiratory enzymes, and those which, like lobeline, are synaptotropic and consequently affect the transmission of afferent impulses. Hollinshead and Sawyer (1945) conclude from the above facts that a chemical agent mediates in the excitation of the carotid body. They suggest that this agent would not be acetylcholine.

Not only are there differences in intensity between the reflex effects of anoxemia and those of central origin, but there are also differences in the speed of the response. This is shown by the frequently cited observations of Heymans, Bouckaert and Dautrebande (1930), of Gemmill, Geiling and Reeves (1934), and of Henderson and Greenberg (1934). These authors observed that the respiration is not increased during the first 4 to 60 seconds of asphyxia due to oxygen-lack produced by breathing pure nitrogen after denervation of the carotid sinus; but that if the asphyxia is continued the hyperpneic reaction appears in the second minute. Carbon dioxide contained in the blood also stimulates the vascular chemo-receptors. In this case however no dominance of the chemo-receptors over the centers is observed. Heymans, Bouckaert and Dautrebande (1930) were able to show that the respiratory response to the breathing of air with CO_2 is produced in the same manner after denervation of the carotid sinus as in the normal state. Gemmill and Reeves (1933); Stella (1935); Wright (1934–36); Gesell and Moyer (1937) demonstrated also intense respiratory reactions to CO_2 after denervation. On the contrary Selladurai and Wright (1932); Schmidt (1932); Green and De Groat (1935); Euler and Liljestrand (1936) observed that with denervation the responses to CO_2, like those to anoxemia, are reduced although never to such a great extent.

It is probable that the differences in the results are due to various factors, the species of animal and the anesthesia. Marshall and Rosenfeld (1937); Comroe and Schmidt (1938); Schmidt and Comroe (1940) believe that the reflexes play a

more important part in anesthetized animals than in those in the normal state precisely because of the effect of the anesthetic on the centers. And they add (1941): "We did not believe that chemo-receptor reflexes are an important factor in maintaining eupneic respiration or in bringing about the respiratory response to carbon dioxide under ordinary conditions." They believe that respiratory centers are more sensible to carbon dioxide than chemo-receptors are.

Gayet, Bennati and Quivy (1935) found intense hyperpnea when the carotid sinus was perfused with equal parts of blood and Locke's solution and the CO_2 tension was increased in the perfusion fluid, but they consider that the effect of CO_2 on the centers is stronger than on the chemo-receptors. Bernthal (1938) and Comroe and Schmidt (1938) state that the reflex effects of the concentration of CO_2 in the blood are less marked, less constant and less well maintained than those produced by anoxemia. Schmidt, Comroe and Dripps (1939) affirm that the threshold of carotid sensitivity to CO_2 is relatively high and always above the threshold of the centers, at least in vagotomized dogs under light anesthesia. Schmidt, Dumke and Dripps (1939–40) confirm this finding; Comroe (1939) points out the existence of marked individual differences in this respect.

Heymans, Bouckaert and Regniers (1933), Gesell (1939) and Schmidt, Comroe and Dripps (1939) agree that great increases in the concentration of hydrogen ions in the blood stimulate the chemo-receptors as strongly as anoxia and more than hypercapnia. Boycott and Haldane (1908) attributed the hyperpnea of anoxemia to an accumulation of lactic acid in the centers. Winder, Bernthal and Weeks (1938) observed an increase in the respiratory movements when the vessels supplying the carotid body were ligated, and after removal of the ligature the hyperpnea and the hypertension disappeared.

Bernthal (1938) perfused the carotid sinus and concluded that in the normal state the carotid chemo-receptors are the origin of tonic vasoconstrictor and respiratory reflexes of great sensitivity which are controlled by the tensions of oxygen and carbon dioxide in the blood. After this Bernthal and Weeks (1939) showed that cooling the blood perfusing the sinus depresses the respiratory and vascular reflexes while warming the blood increases them; they attribute these effects to variations in the acid-base equilibrium of the receptors related to their metabolism. Stadie, Austin and Robison (1927) have already demonstrated the increase in acidity in the tissues as a result of warming.

Schmidt, Comroe and Dripps (1939), and Schmidt, Dumke and Dripps (1939–40) have repeated the experiments of Bernthal using a saline perfusion fluid instead of blood, and carefully determining the concentration of CO_2 and O_2 as well as the pH of the solution. They confirm the results of Bernthal and Weeks but they attribute the effects of the temperature changes to modifications in the gas content of the blood. Winder (1942) perfused the carotid sinus with heparinized blood in Locke's solution which carried CO_2 and O_2 in different concentrations. The effects of hypoxia and of hypercapnia are similar. The chemo-receptors are considered as one of several probable sites for mutual facilitation of hypoxia and hypercapnia acting as stimuli of respiration. Marshall and Rosenfeld (1937) obtained prolonged excitation of the sino-aortic receptors upon in-

jecting pyruvic acid cyanohydrin, probably due to slow, prolonged liberation of cyanide.

The most effective agent for the excitation of the centers and of the chemo-receptors , as Gesell has maintained since 1925, would be a change in the concentration of hydrogen ions in the neurones themselves when the internal environment, the blood or interstitial fluid, becomes acid or particularly when changes in the metabolism flood the neurones with acid metabolites. Warming, ischemia of the sinus or local poisoning with cyanides or sulfides all act in this way. Winder (1942) agrees that intracellular concentration of hydrogen ions is a factor in the control of chemo-receptor activity. Gesell, Krueger, Gorham and Bernthal (1930) examined some time ago the circumstances which can change the concentration of hydrogen ions in the tissues in relation to the insufficient supply of oxygen and the state of their metabolism. They suggest that impaired oxidation leads to increased acidity and that the reverse may also be true.

Bernthal (1938) has shown that anoxia, cyanide, hypercapnia and lactic acid cause reflex peripheral vasoconstriction through their local action on the carotid body, and the vascular reactions are accompanied by respiratory reactions. There is an exact correspondence between the activity of the chemo-receptors and their acidity. Nevertheless the respiratory effects of changes of the hydrogen ion concentration in the carotid body should not make us overlook the sensibility of the respiratory centers to these changes. Moyer and Beecher (1942) assert that decreased oxidations within the center constitute an important factor of respiratory adjustments due to changes in the central pH. This was confirmed by Comroe (1943). He applied upon the respiratory medullary center minute amounts of CO_2-bicarbonate mixtures and observed marked respiratory responses according to the pH of the mixtures. Garcia Banús, Corman, Perlo and Popkin (1944) found that in anesthetized dogs deprived of their chemo-receptor reflexes by the denervation of the carotid sinus and section of both vagi nerves, the respiratory compensation by the centers may be efficient enough to maintain the pH of arterial blood constant at least within 0.01 even if changes of O_2 and CO_2 tension occur in the inspired air.

Gesell (1939) shows that changes in pH in the nerve cells act in the same sense and in the same way in the respiratory centers and in the chemo-receptors: both are sensitive to carbon dioxide and to oxygen lack and hence to externally caused variations in the hydrogen ion concentration, but they are specially sensitive to variations in hydrogen ion concentration of internal origin brought about by the cellular metabolism. As a result of this central and peripheral sensitivity to hydrogen ion concentration, an adjustment is established between the nutritive requirements, the respiration and the circulation.

A characteristic example of these mechanisms of co-ordination is what Hess (1917) has called the "nutritive reflex" which has local and general vascular effects, as Fleisch (1938) and Rein (1938) among others have proved. It is known that any increase in metabolic activity raises the cardiac output. The most effective of the stimuli which give rise to these reflexes of nutritive origin is the acidity of active tissues.

Besides, as Gibbs, Gibbs, Lenox and Nims (1943) wrote, CO_2 improves the oxygenation of the tissues when the O_2 tension in air is low, which enhances the importance of hydrogen ion concentration in cells and in fluids, assuring fine respiratory adjustments from centers to tissues.

Nielsen (1936) suggests in opposition to the idea that the ordinary stimulus of the chemo-receptor centers is the increased local acidity, that carbon dioxide acts by virtue of specific properties and not because it is an acid. CO_2 is said to be the normal stimulus for the respiration: a chemical stimulus produced without interruption in the organism, the principal functional mediator in maintaining the physiological tone of the most diverse functions, especially the circulation and the respiration.

IV. As a result of the simultaneous action on the centers and on the chemoreceptors and as a result of the differences in excitability of centers and receptors depending upon the chemical stimuli acting upon them, various explanations have been developed for the adaptation of the respiration to the changing physiological needs. It is a difficult problem and in certain respects, as it often happens, an imaginary one. The opinions of authors differ in attributing preponderance to the peripheral factors or to the central ones in each case and greater or less physiological significance to the one or the other.

Haldane and Priestley (1935) point out the exquisite sensitivity of the respiratory centers to CO_2, which has long been known. Jongbloed (1936) schematizes the respiratory control stating that carbon dioxide excites the centers directly, while low tensions of oxygen cause tonic excitation of the chemo-receptors, particularly those of the sinus, from which impulses arriving at the centers sensitize them by lowering their functional threshold. Nielsen (1936), Henderson (1938) consider similarly that variations in oxygen to the point of anoxemia control the central activity which is set in motion by the presence of CO_2 in the blood. The tension of oxygen present affects the local action of CO_2 in the respiratory centers.

According to Bernthal (1938) the sensitivity of the carotid body to CO_2 is more variable than its sensitivity to oxygen, and decreases in the presence of an excess of oxygen. The normal tension of CO_2 in the blood exerts a tonic action on the function of the respiratory and circulatory centers by way of the chemoreceptors. Schmidt (1932) stated that the participation of the reflexes from the sinus in maintaining the respiratory movements is to be inferred from the marked reduction or abolition of the respiratory response to anoxemia after denervation of the sinus. But the respiratory reflexes of the sinus are essential only in the case of hyperpnea due to anoxia; apart from this defensive reaction, they do nothing which could not be done without them through changes in the blood flowing through the centers. Sensitivity of the cells of the center to the tension of CO_2 or to the concentration of hydrogen ions in the arterial blood is greater than the sensitivity of the carotid body. The nervous centers take precedence as the most highly specialized part of the mechanisms which control the adjustment of the respiration.

Schmidt insists once again (1941): "The part played by chemo-receptor reflexes in adjusting pulmonary ventilation to the requirements of the body must be negligible under normal conditions. This is indicated by the undoubted facts that the hyperpnea of exercise is not associated with any change in the blood that could stimulate these structures; and that the hyperpnea of carbon dioxide inhalation has not been found to be modified in any measurable way by removing the chemo-receptor influence.... The place of these reflexes in the body's economy lies, not in their sensitivity to factors which ... normally regulate breathing, but in their ability to withstand and to respond under adverse circumstances which seriously interfere with the functional capacity of the central neurons. By virtue of this ruggedness, the chemo-receptors are enabled to set up a powerful reflex drive and thus to maintain the activity of the neurons when the latter have lost their ability to respond to their normal stimuli.

Comroe and Schmidt (1938) consider also that the regulation is brought about by the tensions of CO_2 and oxygen in the blood bathing the centers and particularly by the concentration of hydrogen ions resulting from the equilibrium between the two gases. The chemo-receptors, which are more sensitive to oxygen-lack than to excess of CO_2, operate only when the deviations in one or the other or both of the two respiratory gases in the internal environment are very great and without sufficient effects on the centers.

Schmidt, Dumke and Dripps (1939) have made comparative studies of the sensitivity of the centers and of the carotid body to changes in the tension of CO_2 in the blood. The respiratory movements are not altered significantly nor does the tension of CO_2 in the arterial blood change after denervation of the carotid bodies so long as the animal is breathing sufficient oxygen. The carotid reflexes play an important part only in extraordinary circumstances and not concerned with the fine control of the respiration.

Gesell and Moyer (1937) made an experimental study of the central and peripheral factors which determine the frequency and depth of the respiratory movements. Lack of oxygen in the inspired air, and therefore in the alveolar air, causes a very obvious increase in the ventilation. This increase is not prevented by section of both vagi nor by section of the pulmonary fibres of the vagus and denervation of the carotid sinus, leaving the cardio-aortic region sensitive and active. Denervation of the sinus facilitates rather than prevents the acceleration, but it reduces the tidal volume. On the other hand denervation of the carotid and of the cardio-aortic region leaving the pulmonary vagus intact favors the acceleration produced by oxygen-lack. Complete denervation of the chemo-receptors of the sinus,the cardio-aortic area and the vagal pulmonary area eliminates the hyperpnea due to anoxia. It is evident that the lack of oxygen affects the chemo-receptors more than the centers. Hypercapnia acts in a different way: it causes a type of hyperpnea which is characterized principally by increase in the depth of respiration. The denervation of the vascular chemo-receptors, with or without vagal block, does not cause visible changes in this hyperpnea; this indicates that excess CO_2 exerts a stronger dominant stimulation upon the

centers than upon the peripheral receptors. From this equilibrium between the function of the centers and that of the receptors there results the adaptation to the various circumstances which can influence the respiration.

Schmidt and Comroe (1940) are still concerned with the functions of the carotid and aortic sensory zones. They reach the conclusion that in the case of anoxemia and of poisoning by cyanide or lobeline, the increase in ventilation is due to reflexes of vascular origin, from the sinus or the aorta. On the other hand, when an increase in CO_2 or in the concentration of hydrogen ions in the blood occurs, the central effect predominates. The reflex factor does not become active until almost the maximal response of the centers has occurred. They suppose that the different circluatory chemo-receptors show a distinct sensitivity to stimuli brought by the blood. Some of them might be active constantly in physiological conditions because of their greater sensitivity; the majority however function successively in increasing numbers only when the level of the stimuli is raised. A definite *quantum* of increase in stimulating activity is necessary to evoke a measurable reflex response corresponding to a definite threshold. The problem of whether the most diverse stimulating agents affect all the receptors or whether there is a specific sensitivity for each group of them has not yet been solved. The scheme proposed by Comroe and Schmidt is kept unchanged: the CO_2, the degree of acidity of the blood and perhaps of the nervous tissue, is the central stimulus and is almost without effect on the vascular chemo-receptors. Oxygen-lack on the other hand is the stimulus for the chemo-receptors which begin to function only in emergency. The same authors believe that the status of the hydrogen ion as a stimulus to centers and chemo-receptors is at present still uncertain. It seems quite possible that the respiratory effects of changes in pH (in so far as they are not referable to corresponding changes in CO_2 tension) are due to chemo-receptor reflexes and not to a direct effect of hydrogen ions on the centers.

Dumke, Schmidt and Chiodi (1941) try once more to elucidate the parts played by the peripheral chemo-receptors of the carotid sinus in the respiratory responses to anoxemia and to hypercapnia. With the animal breathing room air, quietly and without exerting any force, the ventilation rate is not decreased by denervation of the sinus, which demonstrates that in such conditions of repose the reflexes from the sinus play no appreciable part. Twelve per cent oxygen causes slight respiratory acceleration, which is increased when the proportion of oxygen in the gas mixture is reduced to 10 per cent. Denervation of the sinus makes the response to the drop in the inspired oxygen much less obvious. If 3.5 per cent carbon dioxide is added to the mixture containing 10 per cent oxygen, the respiration is increased; this increase is slightly less when the sinus is deafferented. From all this the authors conclude that hyperpnea produced by anoxia is reflex in nature, depending upon the stimulation of the vascular receptors, while the presence of an excess of CO_2 acts rather upon the respiratory centers. Gesell and Lapides (1938) performed an interesting experiment: they determined the duration of the apnea produced by over-ventilation of the alveoli in intact dogs and comparatively after blocking the nerve fibres of the sinus with cocaine.

With the nerves blocked the apnea lasts considerably longer. This means that with the afferent nerve of the sinus no longer conducting impulses and therefore with the chemo-receptors no longer functioning, a lower concentration of oxygen is required to excite the centers.

The observations of Witt, Katz and Kohn (1934) lead us to suppose that the participation of the chemo-receptors in maintaining the respiration is not dispensable. Deafferentation of the respiratory centers by section of the vagi and denervation of the carotid sinus results in depression of the respiration and in some cases in cessation and subsequent death. The impulses which normally reach the centers by the afferent nerves stimulate and condition the activity of the centers, and the respiratory mechanism depends to a great extent on the integrity of the peripheral receptors and its conductory nerves. Finally the authors consider that, in addition to the receptors of the sinus and to those which send their fibres through the vagus (pulmonary and cardio-aortic receptors) still others may play a part in the physiology of the respiration.

The centers and the chemo-receptors act in co-ordination in response to and according to the state of the gas content of the blood. Gesell (1939) asks whether there could be transitory and variable differences in the respective sensitivities and whether for that reason there might be produced physiological variations in control according to the circumstances prevailing in the respiration, which are always variable. Thus the threshold of a given chemical sensitivity would not be constant in a given place either central or peripheral.

If during intense hyperpnea by hypercapnia, the nerves of the carotid sinus are blocked with the vagi blocked as well, no decrease in the respiratory movements is observed at the time when the chemo-receptors should be stimulated most strongly. This could be explained in two ways: 1. The respiratory centers respond more vigorously than the receptors to high tensions of CO_2, while the reverse is true at lower tensions. 2. Hypercapnia is unable to maintain the reflexes in this case because the carbon dioxide in the centers blocks the impulses arriving from the chemo-receptors (Gesell and Moyer, 1935). Thus during intense hypercapnia, central stimulation of the vagus produces no effect on the respiration. It seems nevertheless that asphyxia ordinarily increases the effectiveness of afferent respiratory impulses and that the effects of chemical stimulation predominate in the respiratory centers (Gesell, Moyer and Kittrick, 1942).

There remain functional relations of great complexity between the peripheral receptors and the centers and variations would be possible in the relative participation of the mechanisms which control the functions of the centers and of the various chemo-receptors. Any rigid schema which is proposed in an attempt to explain these complicated phenomena can scarcely conform to all the facts.

V. There still remain other important factors which must be considered in studying the action of carbon dioxide on the centers and on the receptors in controlling the respiration. Not only is hypercapnia important, but also a factor of great influence is the lack of CO_2 sufficient to alter its necessary quantitative relationship with oxygen in the air and in the blood.

Mosso (1885) called attention to the effects of hypocapnia when rarefied air is breathed at high altitudes. Since then many have been carried out, some of which have had immediate practical importance. We refer particularly to the work of Yandell Henderson and his collaborators since 1908.

Hasselbalch (1912), Hasselbalch and Lindhard (1915) and Henderson and Haggard (1918) demonstrated that the ratio $\frac{H_2CO_3}{NaHCO_3}$ is altered by hyperpnea (L. J. Henderson, 1919). The concentration of H_2CO_3 in the blood is reduced by the increased expiration of CO_2 and the alkalosis must be compensated by a reduction in base, principally in fixed alkali, i.e., sodium, which is followed by an increased elimination of ammonia, sodium, etc., in the urine. On the other hand a higher concentration of carbon dioxide causes an increase of alkali in the blood. This has been confirmed repeatedly (Haldane, Kellas and Kennaway, 1919; Davies, Haldane and Kennaway, 1920; Gesell, 1922–23; Gollwitzer-Meyer, 1924; etc). Henderson (1925) and Gesell (1925) have summarized in two excellent reviews what was then known of the chemical regulation of the respiration, referring of course at that time only to the action of chemical stimuli on the centers.

When the respiratory elimination of CO_2 becomes excessive, as in the hyperpnea of anoxia for example, a paradoxical situation arises with increased pulmonary ventilation and simultaneous hypocapnia of the centers, in which the CO_2 is washed out. The more intense the hyperpnea of anoxia with subsequent hypocapnia particularly during anesthesia, the less will be the central control and the greater the peripheral control through the chemo-receptors. Hypocapnia reduces the excitability of the centers (Gesell and Lapides, 1938) and if it remains excessive the centers do not provide adequate ventilation. Anoxia results finally in respiratory depression, which is to be explained by the paralyzing effect, especially on the nervous functions, when the oxygen lacks (Verworn, 1903). The nerve cells are particularly sensitive, more so than any other cells, to anoxia and to asphyxia.

The chemo-receptors are in general strongly excited by degrees of anoxemia which depress or paralyze the physiological activity of the central neurones. But so long as the anoxia of the centers is not excessive the lack of oxygen increases their excitability to CO_2, the normal chemical stimulus. In this way the tension of oxygen in the blood would intervene in the control of the ventilation by its action on the respiratory centers. As the anoxia becomes more severe, asphyxia of the neurones hinders the process of regulation, impeding or even paralyzing the central responses; but at that time the chemo-receptors are still able to respond to the lack of oxygen in the blood and the respiration is maintained by the impulses which they set up as a compensatory reaction. This persistence of the excitability of the receptors leads one to suppose that the metabolic level of the sensory cells which compose them is lower than that of the central neurones.

Gesell (1939) holds to his old point of view: the regulation of the respiration is carried out by the acid-base equilibrium, by the pH in the nerve cell, and not by that in its environment, the interstitial fluid, still less by that in the blood. It

is the acid-base equilibrium of the centers primarily and also of the chemo-receptors. Only by taking into account the state of the acid-base equilibrium, not in the internal environment but in the intracellular fluid itself, is it possible to explain a number of facts which otherwise present insoluble problems.

Acidosis of the blood, for example, is not in all cases an obvious cause of hyperpnea. Henderson (1938) has shown that in carbon monoxide poisoning when there is an increase in the ventilation alkalosis is produced by hypocapnia which up to a certain point protects the centers from the harmful effects of anoxia.

We have seen the significance of the accumulation of acids in the cells of the centers and of the chemo-receptors; acids which arise in the intermediate metabolism of the cells and are also dependent upon the acids of the surrounding fluid, the internal environment. Gesell (1925) has explained how the respiratory centers function to maintain their optimal acid-base equilibrium. McGinty and Gesell (1925) have shown that the concentration of lactic acid in the brain and also in the blood increases when carbon monoxide is administered. The same thing happens in cyanide poisoning and in hemorrhage. Local accumulations of lactic acid and of other acids(pyruvic, citric, etc.) are possible in the nerve centers and other organs, in some cases perhaps without any increase in the acids of the blood.

We can explain now the danger of administering oxygen alone in attempting to combat anoxemia. The oxygen releases quantities of acid, particularly lactic acid, which disappears by oxidation and by resynthesis, and free base remains which binds CO_2, decreasing the normal stimulus for respiration. For this reason in respiration at high altitudes the addition of CO_2 to the inspired oxygen helps to increase the oxygen in the blood, to restore normal respiration and to produce the subjective improvement which is felt with each breath, as has been shown by Schneider, Truesdell and Clark (1926), and by many others, most recently by Dill (1938).

The final conclusion from all that has gone before is that for various reasons—lack of oxygen, excess of CO_2, hemorrhage, the action of certain poisons, and increased temperature in the centers or chemo-receptors—the general acid-base balance of certain organs can be changed and it is precisely the state of this balance which controls the respiration. One must not judge this equilibrium only from the pH of the blood; the local intracellular pH of neurones in the centers and of the sensory cells or neighboring cells in the peripheral chemo-receptors is of greater importance. Various factors act upon these equilibria and only by considering exactly the complex influence of such factors can one explain certain facts which otherwise would seem paradoxical if not contradictory.

Schmidt and Comroe (1940) report that many different substances evoke reflexes through their action on the chemo-receptors, particularly those of the carotid sinus. The active substances are of various sorts. We can explain the mechanism of action of some, according to what has already been said, by their influence on the nutrition of receptors through the cellular metabolism; this explanation cannot be applied to others to which we might attribute a

specific action, like that of so many drugs which act on various portions of the autonomic system.

It should be pointed out that the receptors are not only affected by variations in the hydrogen ion concentration, but that they may be excited by a variety of agents foremost among which are normal or accessory products of the intermediate or final metabolism of the tissues. It is possible that there is actually something more in these mechanisms than the regulation of oxidations and the production of acids. It may be that the processes of chemical excitation have greater significance and even though it has not been possible to confirm the mediation of endocrine products of the glomerular tissue in accordance with the hypothesis of Moniz de Bettencourt, Rodrigues Cardoso and Paes de Vasconcellos (1938), it would not be illogical to suppose that the capacity to stimulate the chemoreceptors and the centers extends to various substances of endogenous origin, metabolites or specific substances, "active substances" in the sense used by Demoor. In this way mechanisms of functional correlation and of regulation would be established, as a result of which the respiratory response could be perfectly adapted from moment to moment to the functional metabolic needs, which change rapidly according to the circumstances. Among those chemical stimuli which are able to evoke such mechanisms the most important is the concentration of hydrogen ions in the appropriate tissues.

VI. As long ago as 1876 Latschenberger and Deahna had formulated the hypothesis of the reflex influence of the peripheral circulation on the regulation of the respiration. Recently various investigators have sought by different methods to locate the vaso-sensory zones. Spalto and Consiglio (1886), Hager (1887), Pagano (1900), Siciliano (1900), Brodie and Russell (1900), Mayer, Magne and Plantefol (1920), Frey and Hagemann (1921), Haggard and Henderson (1922), Hess (1923), Tournade and Malméjac (1931), Tournade (1932), Tournade and Rochisani (1934), etc., demonstrated the sensitivity of the vessels and the effects of the chemical excitations which occur in them. The stimulation of these vascular receptors has effects which are predominantly circulatory, but which are inseparably linked to respiratory effects. Among these reflexes particular interest attaches to the respiratory reflexes which are produced by variations in the pulmonary circulation as a result of the stimulation of receptors in the vessels, as observed by Harrison, Harrison, Calhoun and Marsh (1932), Schwiegk (1935) and Christie (1938). Waele and Van de Velde (1940) describe respiratory reflexes through cardiac receptors.

In the same way receptors are found in various organs the excitation of which results in respiratory reflexes. Lewy (1891), Varaldi (1893), DuBois-Reymond and Katzenstein (1901–02), Baglioni (1903), Fleisch (1921), Scott, Gault and Kennedy (1922) have repeatedly confirmed the existence of such reflexes evoked by the excitation of sensory endings of the muscles. The most obvious effect is obtained from the diaphragm and from other respiratory muscles (Fleisch, 1928–30; Hess, 1931; Sharpey-Schafer, 1932; Gesell, 1935). Krogh and Lindhard (1917) produced respiratory reflexes upon faradic stimulation of various groups

of muscles. These effects may be similar to those which are observed when any sensory nerve capable of causing pain is stimulated.

The metabolic state of the tissues and particularly of the muscular system may therefore produce reflexes. It is well known that the respiratory activity increases automatically when the work is increased; this is due to central processes but still more to nutritive reflexes, reflexes which one may suppose are organized to constitute systems,—sometimes very extensive systems,—of respiratory reflexes. Because the increase in respiration begins at the same time if not before the commencement of work, Krogh and Lindhard (1920) thought that this was a matter of central effects, of efferent irradiation in connection with the muscular excitation from the centers (Paterson, 1928). Allen (1942) describes respiratory effects as responses to conditioned reflexes. All this presupposes the existence of reflexogenic receptors in the organs. Harrison, Harrison, Calhoun and Marsh (1932) and Harrison (1939) state as a result of their experiments on dogs and their observations of healthy and diseased human beings that reflexes evoked by movements of the legs help to produce the hyperpnea of exercise. Alam and Smirk (1937) showed the existence of a chemo-sensitive system in the muscles which is capable of evoking vasomotor reflexes as a result of the accumulation of metabolites produced in exercise. Comroe and Schmidt (1943) confirm the possibility of other similar respiratory reflexes resulting in hyperpnea and evoked by movements of the legs. These reflexes are thought to be elicited by excitation of the muscular chemo-receptors which are sensitive to inorganic metabolites, especially carbon dioxide. These mechanisms were suggested long ago by Volkmann (1841) and by Vierordt (1844).

Schmidt and Comroe (1941) and Comroe (1944) assert that in the hyperpnea of exercise the respiratory adjustment depends on receptions in the muscles, lungs (Christie, 1938; Harrison and others, 1939) and perhaps in the heart (Waele and Van de Velde, 1940), besides many other well-known important factors.

The stimulation of chemo-receptors in the active tissues has been conclusively demonstrated as has their effect on the circulation and the respiration. We have referred above to the nutritive reflexes. Through their mediation the cardiac output arises as the metabolic exchange in the tissues is increased.

The regulation of the vegetative functions becomes progressively comprehensive until resulting in generalized responses. Thus, reversing the relationship stated above, respiratory stimuli affect the circulation in an entirely appropriate fashion. The addition of CO_2 to the inspired air increases the blood flow in the brain and decreases it in the muscular system as has been demonstrated repeatedly by Schmidt (1928) in the cat, Lennox and Gibbs (1932) in man, Irving and Welch (1935) in the rabbit, and Irving (1939) in the beaver and the muskrat. Bernthal (1934) observed that when the carotid sinus was perfused with blood containing oxygen at a tension 10 mm. Hg below the normal there is a reduction in the blood flow through the axillary artery. An increase in the CO_2 tension of the blood increases the flow. Section of the vagi makes these effects more obvious.

From all this it is to be concluded that the respiration like the circulation is controlled from the organs themselves by the presence of chemical stimulating substances. We have shown (1941) that a reflex increase in the respiratory movements is produced by asphyxia of the trunk when it has been isolated from the head and separated therefore from the carotid sinus and when the heart has been denervated, inactivating the cardio-aortic chemo-receptors. This effect can only be the result of the stimulation through CO_2 of receptors located in the lungs or in the tissues or in both regions.

VII. Afferent impulses of the most diverse origin produced by the excitation of receptors of all sorts reach the respiratory centers. Besides stimulation of mechanic and chemo-receptors, the stimulation of other more or less specific receptors is important. In the first place we may note the stimulation of many sensory nerves which evoke respiratory reflexes. Painful sensations can retard or accelerate the respiratory movements depending upon the circumstances (Henderson, 1910, Meyer, 1914). Proprioceptive sensations coming from muscles and proprioceptive sensations of position from muscles, joints and labyrinths affect the respiratory movements. Stimulation of the special senses of sight, hearing and smell and of the sensory endings for cold and heat result in changes in the respiration. This is true also of impulses from the autonomic regions. Centripetal impulses which affect the respiration may come from the whole organism by all pathways.

One must not forget on the other hand the automaticity of the respiratory centers which can maintain up to a certain point alternate inspiration and expiration in the absence of any external influence. This was suggested by Legallois (1812), and recognized later by a large number of investigators (Langendorf, 1888; Schrader, 1887; Lewy, 1891; Lewandovski, 1896; Foa, 1909–11; Winterstein, 1911; Scott and Roberts, 1923; Roberts, 1925; Adrian and Buytendijk, 1931; Finley, 1931; Barcroft, 1934, and many others).

The anatomical localization of the respiratory centers and their functioning have been studied intensively. Flourens supposed that they are located near the tip of the "calamus scriptorius," and Gierke that they are made up of cells connected to the fasciculus solitarius. Mislavski (1885), Aduco (1890) and recently Henderson and Sweet (1929), Finley (1931), Henderson and Craigie (1936), Nicholson (1936), Nicholson and Brezin (1937), Nicholson and Sobin (1938) search the localization of the respiratory centers in the medulla. Worthy of special comment is the paper by Gesell, Bricker and Magge (1936) who studied the electric changes in the function of the medullar respiratory centers. These authors, like Brookhart (1940), state that the neurones which make up the bulbar centers are found more or less scattered through the reticular formation. Pitts, Magoun and Ranson (1939) and Pitts (1940–42) attempt to give a precise anatomical and physiological description of these myelencephalic centers. They distinguish in the cat an "inspiratory area" situated in the ventral reticular formation below the inferior olive, and another dorsal, "expiratory area", somewhat higher, extending beyond and bending over the cephalic portion of the inspiratory area. Excitation of one or the other gives rise respectively to contraction of the muscles of inspiration or of expiration.

There are different physiological categories of centers, the higher centers integrating the function of the lower. This is known since the researches of Markwald (1887–90), Loewy (1888), Langendorf (1888), Luscher (1899), Lewandovsky (1896), etc. Thus Lumsden (1923) and later Stella (1938–39), among others, thought that there is a regulator center in the pons—"pneumotaxic"—which has predominant inhibitory properties and so it is able to control the function of the other subaltern centers. Rijlant (1932), who studied the efferent respiratory electric discharges, established a functional hierarchy among the respiratory centers, possessing different specific properties and located in the medulla and the pons. These centers are not compact nor do they form a strict entity but rather a system of adjacent groups of neurones among which those which have thus far been localized experimentally are of particular functional importance.

In the centers the impulses of various origins are integrated and organized. Motor patterns are formed through the synthesis of the most numerous and various elements and these patterns or functional systems give rise to efferent impulses to the muscles and also to other higher centers both subcortical and cortical, which work in unison and in perfect adjustment with the bulbo-pontile centers.

For a long time it has been supposed that the inspiratory-expiratory alternation was a sequence of reflexes started at the vagal ends of the lungs and related to mechanical stimulus: the state of pulmonary inflation or deflation. The discovery of the Hering-Breuer effect (1868) led to a conception universally accepted. The explanation appears by now incomplete. Expiration, like inspiration, can be active. And side by side with the mechanical stimulation of peripheral receptors should be considered the function of chemo-receptors in different territories, and the intrinsic properties of the respiratory centers at various levels of the neuro-axis, especially the medullo-pontile centers.

Bronk and Fergusson (1935) employ the inscription of the electric "fusillades" by the motor nerves to study this question. They conclude that both inspiration and expiration are active and that the rhythmic impulses of excitations arise automatically in the centers, even in the absence of afferent impulses arriving to them. Gesell (1940), Gesell, Magee and Bricker (1940) and Gesell, Atkinson and Brown (1940) describe the patterns of the electric variations in the respiratory motor nerves; inspiratory impulses present there different patterns from the expiratory ones. These experiments show also that the centers are active in inspiration and expiration and that centrifugal currents may leave such centers independently of any afferent impulses. In the respiratory mechanisms there are centrogenic excitations beside reflexogenic influences. The centers maintain a tone and send out rhythmical impulses over the centrifugal pathways; but they are subject to numerous afferent sensory influences of varied origin and nature, and subject also to their own chemical state and that of the blood passing through them.

Gesell and Hamilton (1941) have made a further study of the reflex mechanism by stimulating three types of nerves: the vagus which they consider proprioceptive, the carotid sinus nerve, chemoceptive, and the saphenous, nociceptive; they

confirm the opinion that the two forces which control the respiration are the activity of the central neurones and the influence exerted on the function of these neurones by afferent impulses arising in receptors of various sorts. The sum total of the influences both central and peripheral which at times interfere and at other times re-enforce each other constitutes the power which drives the central nervous system. An overall summation of these different influences is brought about, an organization which the authors attempt to schematize and by means of which the nervous control of respiration is explained. Gesell and Atkinson (1943) deal with the "motor integration" of the respiration still using the analysis of the respiratory discharges in the diaphragm, in various species of animals. In the centers elementary phenomena are integrated which may possibly be identical or similar along the zoological scale and which become complicated in various ways according to the level of function.

Pitts, Magoun and Ranson (1939) study the functional interrelation of the different respiratory centers in the cat employing the Horsley-Clarke technique. There are in the medulla inspiratory and expiratory bilateral groups of neurones, and fibres of the vagus are connected with them. Pitts (1942) reaches the conclusion that four subsidiary systems are present in the medulla: a, an excito-motor center which sends out periodic discharges; b, a vagal inhibitory system; c, a cerebral inhibitory system, cortico-hypothalamic; d, other excitatory and inhibitory systems. The inhibitory systems, both vagal and cerebral, check the activity of the neurones of the motor system and in this way rhythmical respiration is produced; it is also possible that they vary the rate and depth according to need.

Comore (1944) writes recently: "Probably the most important discovery of the last century has been the realization that respiration is controlled not by stimulation of the medulla alone, neither by reflexes alone, but by proper interaction of both factors. No reflex, no matter how strong, can stimulate respiration if the arterial CO_2 tension has been lowered abnormally (Krogh and Lindhard, 1913; Stella, 1939); no chemical stimulant, no matter how great, can produce rhythmic breathing if the medullary centers have been completely cut off from all nervous influence including that residing in pneumotaxic center (Stella, 1938). Respiratory alterations in general cannot be explained by a single theory but only by a consideration of a number of known and probably many unidentified factors. This has been our thesis, a long time ago, since our initial researches.

We cannot finish without stressing the participation of certain cortical regions in maintaining and adapting the respiratory movements to physiological requirements. Consider for example the adaptation of the respiration to the requirements of spoken language. It is not surprising that speaking as well as silent reading (Bellido, 1922), rhythm (Japelli , 1906; Coleman, 1920), various psychic effects (Allen, 1929–42), emotion and finally consciousness and the will all influence the respiratory movements. Fulton (1943) asserts that the regulation of the respiratory movements is a problem of general physiology in the widest sense. A broad concept of the function will keep us from attributing excessive importance to a few isolated facts and from concluding that extremely simple

and limited mechanisms, such as reflexes divorced from the function of the whole, can determine by themselves the flexible respiratory dynamics. Everything which leads to the recognition of new facts is important. But let us not advance an explanation of the whole, which depends upon innumerable factors, in terms of one elementary function—one receptor, one center and one effector. Investigations which have been carried out without a logical conception of the totality of the neural organization and of the adaptation of the responses have contributed to the obscurity of the conclusions and to a general disorientation. The physiologist must be a careful experimenter, but he must have above all a clear idea of what he is seeking; he must attribute to his observations their exact significance and he must know how to incorporate his results into the general theory.

Therefore when we contemplate in the proper perspective the history of the fundamental discoveries in the physiology of respiration we are surprised at the extreme pains taken by some authors to deny the existence and function of chemo-receptors in the respiratory apparatus. Our purpose in studying this problem was motivated by a leading idea expressed in the thesis of Turró (1914), which stated the functions of a "trophic sense" of chemical nature possessed by many different tissues. After the demonstration of the trophic reflexes of hyperglycæmia (Pi-Suñer, 1917) and hyperlipemia (Geelmuyden, 1923; Wertheimer, 1926), it seemed to us that it would be an interesting problem to determine whether a reflex mechanism responsive to peripheral chemical stimuli also acts to control the respiratory movements, which are so easily observed and which offer so much for investigation. The results were positive and the researches of numerous investigators have given a decisively favorable response. From the nose (Allen, 1929; Kerekes, 1935; Deseo and Fodor, 1935) and the larynx and trachea (Lumsden, 1924; Graham, 1939–40) to the tissues themselves," a series of chemo-receptors takes part in the control of the respiratory movements: in the respiratory apparatus at various levels, in the circulatory systems and in other parts of the organism.

The vascular chemo-receptors act effectively in the regulation of the respiratory movements but it cannot be doubted that the location of the chemo-receptors in the respiratory apparatus, at various levels, from the nose to the finest bronchioles and the lungs, is a more strategic one. It is logical to suppose that in mammals, animals which breathe air, receptors in the respiratory apparatus itself have greater functional significance than have vestiges of sensory structures in the vessels, which are useful primarily for respiration in the water (Schmidt, 1938).

Gesell wrote recently: "If ever there was a conviction firmly intrenched in physiology, it was the monopoly of the chemical control of breathing by the respiratory center. . . . *It proved to be one of physiology's outstanding creeds.* . . . It was a shaky foundation upon which all of us worked. So when Heymans, *et al.*, produced hyperpnea by a lack of oxygen or an excess of carbon dioxide confined to the aortic (1924–27) and carotid chemo-receptors (1930–32), *he gave us a new outlook on respiration* for which physiology is deeply indebted. . . . *Both central and peripheral* chemical control were proven to be extremely important."

For over a quarter of a century we have been investigating the functions of the respiratory chemo-receptors. The idea of the rôle of the peripheral chemical stimulation of receptors in the processes of regulation of pulmonary ventilation and respiratory dynamics was enunciated by us in 1918 and at the same time the reflex effects from those stimuli were demonstrated. That year we published our first paper on chemo-receptors in the respiratory apparatus, which control its motor functions, and in 1920 we gave a practical demonstration of the technique employed (the dog with two heads, perfusing the head of the experimental dog with the blood of the donor dog) before the Tenth International Physiological Congress in Paris. Since those first experiments, our research work on this subject has been extensive. Today it is an undisputed truth that *peripheral chemo-receptors* are instrumental in the control and the regulation of the respiratory movements.

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THE ACTION OF INSULIN

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In spite of the enormous amount of both experimental and critical work devoted in earlier years to the problem of insulin action, and in spite of the apparently convincing demonstration furnished by earlier work that insulin facilitates both glucose oxidation and storage as muscle glycogen, too many facts are still disputed, too many conclusions remain questioned, to allow a satisfactory agreement to be reached on the subject. Since then, a great step forward has been made, thanks particularly to the results achieved by Soskin and other American authors; new light has been thrown simultaneously on the biochemical aspect of the problem by the Coris' more recent work on glucose and glycogen phosphocatalytic metabolism. To these important results we now wish to add our own contribution to the subject.

It may be recalled that the problem of insulin action has been studied uninterruptedly since as early a date as 1923, in the physiological laboratory at Louvain University. The earlier experiments by Bouckaert, De Nayer and their coworkers already pointed against the current views on the subject; as to our later results, not only do they conclusively disprove the classical muscular theory of insulin action, but, jointly with Soskin's and other recent data, they afford sufficient evidence for reconsidering the whole bulk of experimental work on which it is based. Out of the new facts thus brought to light, a different theory arises, which provides a more comprehensive and satisfactory picture of insulin action than has yet been put forward. As has been shown in the detailed review on carbohydrate metabolism, diabetes and insulin, recently published by one of us (27), practically all the results obtained until now on this subject may be gathered, when properly interpreted, in support of this theory.

Our essential aim, in this paper, is to summarize our most important results and to point out briefly how they affect the general theory of insulin action. For a more detailed account we wish to refer to the work of de Duve (27), already mentioned, where the more recent literature has been extensively discussed.

EXPERIMENTAL RESULTS AND DISCUSSION. a. *The importance of the blood sugar level.* Some experiments by Wierzuchowski, Soskin, Lundsgaard, amongst others, have served to emphasize a relationship between blood-sugar level and blood-sugar consumption. Such a relationship has been shown to exist by De Nayer (17) between the amount of glucose deposited as muscle glycogen under insulin action and the blood-sugar level at which the insulinized animal is maintained by continuous intravenous infusion of glucose; we ourselves have observed the quantity of glucose disappearing under the influence of insulin to be directly proportional to the mean glycaemia of both rabbits and dogs receiving insulin and glucose intravenously (19).

The mathematical function relating blood-sugar consumption to blood-sugar level has been the object of some disagreement. According to Lundsgaard and

co-workers (44) the function is linear in a range of blood sugar from 0.20 to 0.60 per cent; Wierzuchowski (64), Soskin and Levine (60) both find the curve to be parabolic. In the latter work, however, the total carbohydrate utilization is considered and not the blood-sugar disappearance only. On the other hand, the data obtained by Wierzuchowski can be satisfactorily put on a straight line in the blood-sugar range in which Lundsgaard finds the relationship to be linear. Our own results show that the linear relationship still appears at lower blood-sugar levels in the case of the insulinized animal. From this fact it may be assumed that the relationship really is linear, but that the curve tends to become parabolic at low or very high blood-sugar levels. This would be due, in the latter case, to saturation of the enzymes responsible for glucose disappearance, in the former to the fact that hepatic glycogenesis[1] replaces to some extent infused glucose in compensating peripheral consumption, and that the exogenous supply, by which consumption is measured, must be accordingly restricted.

The relationship between *glucose administered* and blood-sugar level would then become linear from the moment when hepatic glycogenesis became completely inhibited, in other words from the hepatic threshold[2] upwards, to the point where the curve is again flattened by saturation of the enzymes. This is indeed the case in Wierzuchowski's experiment, if we assume that, in accordance with Soskin, the normal value for the hepatic threshold lies between 0.15 and 0.20 per cent. Our own results would then support the view that insulin considerably lowers the hepatic threshold, allowing the real relationship to be observed at much lower rates of glycaemia, where the curve of the non-insulinized animal already assumes a parabolic aspect. As will be shown, this view is conclusively demonstrated by our further experiments.

To our knowledge, no explanation has yet been put forward to account for the observed relationship between blood-sugar consumption and blood-sugar level. We ourselves consider it as a *necessary consequence of the law of mass-action.* Whatever the tissues concerned and whatever the final product of this transformation may be, glucose consumption must necessarily be equivalent to glucose transformation and must therefore be due to chemical reactions. The *initial reaction*, by which glucose enters the metabolic processes will occur at the expense of glucose itself; *its velocity will be increased by an increase in the concentration of glucose.* Secondly, the subsequent reactions will also be accelerated and a total increase of the metabolic turnover of glucose will occur. In accordance with this

[1] To prevent confusion, we wish to point out that, throughout this paper, we have used the expression "hepatic glycogenesis" in the sense of "sugar formation by the liver," whatever the origin of the sugar formed. Hepatic glycogenesis will therefore cover both "hepatic glycogenolysis" (sugar formation from glycogen) and "glyconeogenesis" (sugar formation from non carbohydrate sources such as protein and, possibly, fat). Synthesis of glycogen will be referred to as "glycogen formation." It should be further noted that the word "glycogenolysis" really means "glycogen breakdown." When applied to the liver, it usually means "glycogen breakdown to glucose;" but many authors also refer to "muscular glycogenolysis" in relation with glycogen breakdown to lactic acid. In the latter case, we have adopted the expression "glycogen breakdown."

[2] The hepatic threshold is the value of the blood-sugar level, at which sugar formation becomes completely inhibited in the liver and gives place to sugar retention.

line of reasoning, the mathematical function relating blood-sugar disappearance to blood-sugar level should have a specific significance, a linear curve indicating that the initial reaction is monomolecular. We will refer later in this paper to the exact nature of this reaction; but it may be mentioned that many facts suggest that the initial process might be a change in the molecular architecture of glucose, brought about by adsorption to some specific enzymatic structure and prior to phosphorylation by adenosine-triphosphate. Such a reaction would indeed be of the monomolecular type; moreover, the process at this stage would still very probably be reversible, which would account for the phenomenon of "*stockage lacunaire*" observed by Soula and his co-workers. Nevertheless, too much significance should not be attributed to the linearity of the curve; as we have seen, it occurs only in a definite blood-sugar range and any limited portion of a parabolic curve is approximately a straight line. On the other hand, the fact that enzymatic processes are conditioned by adsorption to a surface renders kinetic deductions from the type of curve open to criticism.

The importance of this relationship in regard to insulin action is evident. It shows that the rate of blood-sugar level should be measured regularly, and the automatic influence of its variations should be taken into account (see later: The method of compensation (p. 43)).

Blood-sugar variations can affect the experimental results obtained with insulin in yet another way. In 1934 attention was drawn by Macleod (46) to the extraordinary differences existing between normal and diabetic animals in regard to their responses to insulin. While the metabolic changes brought about by insulin in diabetic animals are fairly regular and may be called "normal", i.e., opposite to the corresponding changes caused in normal animals by total extirpation of the pancreatic gland, the reactions of the normal animal to insulin are much more irregular. In a certain number of experiments, the response is also "normal," in others no changes occur, in many others, finally, "abnormal" diabetic reactions to insulin are observed, for instance a lowering of the R.Q., a decrease in hepatic glycogen, an increase in the production of ketone bodies, or a rise in nitrogen excretion, etc.

Macleod failed, however, to give a satisfactory explanation of these differences. There is no doubt that they are mainly due *to differences in blood-sugar level.* A systematic review of those earlier experiments, and of the later ones where similar differences occur, has shown us that most of the "abnormal" results were obtained on animals receiving large amounts of insulin and no glucose or insufficient quantities of glucose, while the "normal" results are observed on either diabetic animals, or on animals receiving only small doses of insulin, or adequately supplied with glucose whenever greater quantities of insulin were given. In other words, *"abnormal" results are generally associated with hypoglycaemia, "normal" ones with hyperglycaemic or normal blood-sugar levels.* In many cases, this relationship appears in the same set of experiments. From this fact may be concluded that the "abnormal" results are mostly due to the action, not of insulin itself, but of *blood-sugar raising or diabetogenic factors whose appearance in the blood-stream is induced by the hypoglycaemic state brought about by insulin.* For this reason

marked hypoglycaemia must be considered as the *chief cause of error* in experiments on insulin and should be avoided at all costs.

In short, the blood-sugar level appears to be of primary importance in all experiments concerned with insulin. We believe that too much stress cannot be laid on its importance, and that the confusion still existing amongst different schools, and the resulting failure of any theory of insulin action yet proposed to carry unanimous agreement, are to be attributed mainly to neglect of this factor, or insufficient knowledge of its influence. As has been shown, this influence is twofold:

1, the rate of glucose consumption is directly related to its concentration in the blood; 2, low blood-sugar levels may induce secondary reactions which antagonize the action of insulin.

b. *The so-called "glycogenolytic action" of insulin.* It has been frequently stated, especially by French and American authors, that insulin exerts a glycogenolytic influence on the liver. In fact, a decrease in liver-glycogen after injection of insulin has been frequently observed; but so, for that matter, has an increase. Of the two results, the former undoubtedly may be termed "abnormal" in view of the diminution in hepatic glycogen reserves resulting from pancreatectomy. In no other set of experiments does the "glycaemic criterion" prove to be such a valuable asset as in these: it may be shown that many of the experiments in which glycogenolysis occurs are associated with hypoglycaemia and are therefore without demonstrative power; it has even been shown in some experiments (see Reid (56), Crandall and Cherry (14)) that glycogenolysis *fails to occur* in adrenalectomized or adrenal-denervated animals treated with insulin, thereby clearly demonstrating that this effect is due, not to insulin action but to a secondary discharge of adrenalin, induced by insulin-hypoglycaemia.

In some cases however, we either have no positive proof that such a reaction has occurred, as no recording of the blood-sugar curve has been made (e.g., Cori and Cori (13)), or may even definitely eliminate the possibility of such a reaction, as, for instance, in the experiments by Bodo and Marks (2), Fiessinger *et al.* (29), Lundsgaard *et al.* (43), where a glycogenolytic action of insulin is demonstrated on *the perfused liver.* An interesting fact about the latter experiments is that this effect on the perfused liver can be produced *with certain samples of insulin only;* others, at least equally active therapeutically, exert no glycogenolytic action whatsoever on the perfused liver. Evidently, even in these cases, glycogenolysis cannot be due to insulin itself; it must be attributed to a blood-sugar raising factor, different from insulin, which is present as an impurity in certain samples of commercial and even crystalline (Fiessinger, Lundsgaard) insulin. It will be remembered that a similar explanation accounts satisfactorily for the initial hyperglycaemia which occurs immediately after injection of certain samples of insulin.

Our attention was particularly drawn to this problem by a paper by Bridge (8), where it is categorically stated that insulin inhibits glycogen formation in the liver. In Bridge's experiments, addition of varying amounts of insulin to the solution of glucose infused continuously to rabbits, during 6 hours and at such

a rate as to maintain the blood-sugar at a constant hyperglycaemic level, leads to a corresponding *decrease* in the amount of glucose deposited as liver-glycogen. The insulin used in this experiment was "*Lilly;*" since this brand has been widely used for research purposes, we thought it rather important to verify whether it also contains a blood-sugar raising factor, as Bridge's results strongly suggested. Our experiments fully confirmed this assumption (24) (26).

Injection of "*Lilly*" insulin produces a characteristic initial hyperglycaemia; less glucose is needed to compensate the hypoglycaemia induced by massive injection of 30 units of another brand of insulin ("*Novo*") to rabbits, when "*Lilly*" insulin is added to the glucose solution; on the contrary, addition of "*Novo*" insulin to the glucose infused leads to an *increase* in the glucose requirements of the animal. When sufficiently high doses of "*Lilly*" insulin are added (16 to 23 U/Kg./hr), *no glucose at all is needed;* the blood-sugar level never falls below 0.09 per cent. The glycogenolytic factor is then supplied at a rate sufficient to activate the hepatic output to such an extent as to replace almost completely a continuous glucose infusion of 1,40 g./Kg./hr. From the quantitative data obtained, it may be calculated that Bridge would have observed an *increase in hepatic glycogen, proportionate to the amount of insulin given,* if he had used another brand of insulin and if he had further maintained all his animals *at the same blood-sugar level,* by adequately regulating the glucose infusion.

We feel that we may safely conclude that insulin itself exerts no glycogenolytic effect whatsoever; on the contrary, provided sufficient glucose is supplied to maintain the blood-sugar at a normal level, hepatic glycogen will regularly be found to increase under the influence of insulin. Further objections which might be brought against this conclusion will be met later in this paper (see "The final fate of glucose disappearing under insulin action (p. 52)).

c. *The method of compensation.* Apart from the fact that the brand of insulin used must be tested as to the possible presence of a blood-sugar raising impurity, the most important factor which should be kept in mind in all experiments concerning insulin action is the level of the blood-sugar. Unless one wishes to study the reaction to hypoglycaemia, low blood-sugar levels must be prevented from occurring; moreover, the direct influence of blood-sugar concentration on blood-sugar disappearance must be taken into account. The most perfect method will therefore be the one in which the blood-sugar is continuously maintained at its normal level by an adequate infusion of glucose. Already in 1929 such a method was devised by Bouckaert, De Nayer and Krekels (4) (5), and it has been widely used subsequently by Bouckaert and his co-workers. Lately, de Duve and Bouckaert (19) have pointed out that the original method can be rendered more serviceable and more precise by taking advantage of the linear relationship existing between blood-sugar level and consumption. In both methods the insulin is given massively by intravenous injection at the beginning of the experiment and this injection is immediately followed by a continuous intravenous infusion of isotonic glucose; it can be shown that if the blood-sugar variations are not excessive, the quantity of glucose infused corresponds, with less than 5 per cent error, to the quantity which has effectively disappeared under insulin action and

provides a very accurate measure of this action. At least, this will be true if the independent and automatic influence of the blood-sugar level itself is taken into account. This was done in the original method by retaining only those experiments in which the blood-sugar curve constantly remained within "physiological limits"; a large number of experiments had thereby to be discarded and the method proved onerous and unpractical. In the new method, the mean blood-sugar level G is calculated from the blood-sugar curve, and D being the quantity of glucose given (i.e., consumed at a mean blood-sugar value G), the quantity D_0 *which should have been given* to maintain a normal blood-sugar concentration G_0 is calculated by the experimentally established formula:

$$D_0 = D \times \frac{G_0}{G}$$

Through this simple mathematical process, the range of blood-sugar curves which remains of practical use is far greater, and the measure of insulin action is far more accurate as all the results are reduced to an identical blood-sugar level G_0, the independent influence of the level of the blood-sugar being thereby eliminated. When assays of this type are performed on a set of similar animals, the values of D_0 usually differ from one animal to the other and can be represented by only a mean value. Finally, the mean value of D_0 thus obtained provides a quantitative test of the action of a given dose of insulin on a given type of animal.

Another modification of the original method has been devised by De Maeyer and De Graer (16). In this second method, the values of G are plotted against the values of D, and the most probable curve relating D and G is calculated. The intersection of this curve with a vertical line passing through the value of abscissa G_0 furnishes directly the most probable value of D_0. The two methods give almost identical results.

It is to be noted that both these methods imply the use of statistical formulae. It is very important, especially in biological research where the number of experiments is usually small, that statistical results should be presented properly, that is, accompanied by their dispersion. This can be done with the aid of the formulae given by Fisher (30) in his book, *Statistical methods for research workers* (see also (16) (19) (27)).

d. *The site of insulin action.* For more than 50 years, two theories have been opposed to explain the origin of diabetic hyperglycaemia and of insulin hypoglycaemia. According to one theory, the main site of insulin action is the liver and diabetic hyperglycaemia is mainly due to overproduction of glucose in this organ, while insulin hypoglycaemia is essentially a result of an inhibitory action on liver glycogenesis. According to the other, insulin acts mostly on the muscles, non-utilization of glucose being the cause of diabetic hyperglycaemia, excessive utilization that of insulin hypoglycaemia. In this second theory, it is assumed that the intense variations in hepatic metabolism which undoubtedly occur in diabetic animals are mainly due to secondary reactions provoked by the primary glucose need of the muscles.

Up to 1930, the muscular theory was advocated by a majority of authors and is still widely accepted today. The arguments for it are manifold: 1. While the fact that insulin acts directly on the glucose consumption of muscle tissue was firmly established by a great number of authors, none of the experiments carried out to reveal a similar action on the isolated liver had given conclusive results. 2. While, on the whole animal, changes in muscular metabolism were fairly regular after administration of insulin, hepatic responses to insulin proved excessively variable and were even often "abnormal," i.e. similar to those occuring after pancreatectomy. 3. Some well known experiments, namely those by Mann and Magath, Lesser and co-workers, Cori and Cori, Best, Dale, Hoet and Marks appeared particularly demonstrative and have been frequently quoted in support of the muscular theory.

Since then, the hepatic theory has gained ground thanks to the work of Soskin and his co-workers; nevertheless, none of Soskin's experiments conclusively disproves the hypothesis of an indirect action of insulin on the liver.

In order to measure quantitatively the amount of glucose which disappears respectively in the liver and in the peripheral tissues under the action of insulin, we have carried out comparative tests, using the method described above, on whole dogs, on hepatectomized dogs, eviscerated dogs and pancreatectomized-hepatectomized dogs, either receiving super-maximal doses of insulin or receiving no insulin at all (de Duve et al. (1945) (20)).

Our results show not only that insulin may inhibit completely hepatic glycogenesis in the animal with a normal blood-sugar but may even further lower the hepatic threshold, thereby considerably increasing glucose retention by the liver. They prove that insulin acts directly on the liver and that the hepatic action is more imporant than the peripheral action.

Here is a more detailed description of the procedure followed in these experiments:

Using the method described in the preceding chapter, we have ascertained that, to maintain a normal blood-sugar level in a dog constantly during 80 minutes after he had received a super-maximal dose of insulin given intravenously, it is necessary to provide, by continuous intravenous infusion, a total quantity of glucose equal to 2,10 gr./Kgr. From this result may be gathered that the various tissues of a dog saturated with insulin remove from the blood-stream large quantities of glucose corresponding to 2,10 gr./Kgr./80 min. provided the blood-sugar level itself does not vary. It will be remembered that if the rate of glucose infusion is such that variations of the blood sugar level occur, this would, under the same conditions of insulin excess, affect the quantity removed, this quantity being larger than 2,10 gr./Kgr. if the dog becomes hyperglycaemic, smaller if hypoglycaemia occurs. We may further assume that, in this experiment, the action of insulin is observed as much as possible uncomplicated by other factors, such as adrenalin and other blood-sugar raising hormones, whose secretion would be increased if the animal were allowed to become hypoglycaemic.

The point we wanted to investigate was the behaviour of the liver under those conditions. We know that the liver of the fasting dog secretes into the blood

stream a quantity of glucose covering the total consumption of the peripheral tissues. We know also of three factors which may alter this process: adrenalin will stimulate sugar formation in the liver by activating glycogenolysis; pituitary and cortical hormones will stimulate it also, mainly by promoting glyconeogenesis; finally, blood-sugar level variations will directly affect the hepatic glucose output, hypoglycaemia leading to its increase and hyperglycaemia bringing about an inhibition of this process, an inhibition which may be partial, or complete, or may even give place to the opposite process of retention, according to the intensity of the hyperglycaemia obtained. In our experiments, changes in blood-sugar level are avoided so far as this is possible; on the other hand, we have no reason to believe that important alterations take place in the secretion of the adrenal and pituitary glands, since their most powerful stimulant, which is the blood sugar level itself, suffers no great variations.

The most important factor obtaining under these experimental conditions will be therefore the considerable excess of insulin to which the animal is submitted, and these conditions appear particularly suitable for the study of the hepatic reaction to this hormone.

If we assume that insulin does not at all affect hepatic metabolism, we must expect the hepatic glucose output to continue undisturbed and the quantity of glucose thus poured into the bloodstream will add itself to that given by infusion to meet the requirements of the peripheral tissues; in that case the value of 2,10 gr./Kgr. should indicate the difference between the peripheral requirements of the animal saturated with insulin and those of the animal receiving physiological amounts of insulin from its own pancreas. If insulin does promote sugar formation in the liver, the excess of glucose thus provided would have to be added to the quantity infused, the sum of the three being equal to the peripheral requirements of the insulinized animal. On the other hand, if insulin partially inhibits sugar formation in the liver, the peripheral requirements of the insulinized animal will be less than the normal fasting glucose output plus 2,10 gr./Kgr., but they will still be larger than 2,10 gr./Kgr.; if insulin completely inhibits sugar formation in the liver, the peripheral requirements of the insulinized animal will be exactly 2,10 gr./Kgr.; and finally, if insulin not only inhibits sugar formation in the liver but further promotes hepatic retention of glucose, the peripheral requirements of the insulinized animal will be smaller than 2,10 gr./Kgr. by a quantity equal to that taken up by the liver under the influence of insulin.

It is clear than an adequate measurement of the *peripheral glucose requirements* of an animal saturated with insulin will allow us to distinguish between these various hypotheses. We have therefore determined the quantity of glucose which must be furnished by continuous infusion to maintain a constantly normal blood sugar level on a number of hepatectomized and totally eviscerated dogs receiving a super-maximal dose of insulin. We have also carried out similar determinations on hepatectomized, on totally eviscerated and on pancreatectomized-hepatectomized dogs, receiving no insulin.

The various results obtained are summarized in table 1.

The most significant results are those obtained on hepatectomized and evis-

cerated dogs receiving large amounts of insulin; they show that exclusion of the liver strongly diminishes the glucose requirements of the insulinized animal and enforce the conclusion that insulin given in large amounts to an animal whose blood-sugar level is kept normal by a continuous infusion of glucose, not only completely inhibits the hepatic glucose-output, but even considerably activates glucose retention by the liver, the amount of glucose taken up by the liver under those conditions (2,10 − 0,40 = 1,70 gr./Kgr./80 min.) being much larger than the total amount taken up by all the other tissues (0,40 gr./Kgr./80 min.).

A more detailed discussion of the results serves to emphasize this conclusion. The results obtained on the hepatectomized animals receiving no insulin give us an idea of the state existing in the normal fasting animal supplied with insulin from its own pancreas. Its peripheral glucose requirements are 0,32 gr./Kgr./80 min.; there is no indication that hepatectomy diminishes the glucose consumption

TABLE 1

NUMBER OF EXPERIMENTS	OPERATIVE DEPRIVATION	INSULIN GIVEN	INSULIN AVAILABLE	GLUCOSE REQUIREMENTS TO KEEP THE BSL CONSTANTLY AT ITS NORMAL VALUE OF 0,87 GR. P. L. GR./KGR./80 MIN. $\bar{x} \pm T\, s/\sqrt{n}$ *	
7	None	+	Super-max.	2,10 ± 0,24	
6	Hepatectomy	+	Super-max.	0,40 ± 0,12	0,40 ± 0,08
4	Evisceration	+	Super-max. and under-max.	0,40 ± 0,22	
3	Hepatectomy	−	Norm. endogenous	0,32 ± 0,06	
3	Evisceration	−	Remaining in tissues immediately after evisc.	0,18 ± 0,15	
2	Previous pancreatectomy hepatectomy	−	None	0,09 and 0,14	

* $\bar{x}$: mean. s: standard error of mean. n: number of experiments. T: "students value of t for P = 0.05 and n values. In less than 5 per cent of cases shall the true value of $\bar{x}$ fall outside the limits indicated by the value affected by the sign ± (Fisher p. 111).

of the peripheral tissues, since this figure is not smaller than those obtained by other authors, some of them on intact animals.[3]

We may take it, therefore, that the peripheral tissues of the normal fasting animal take up 0,32 gr./Kgr./80 min. of glucose from the blood-stream, the same quantity being poured into the blood-stream by the liver. In other words, the liver supplied with physiological amounts of insulin secretes 0,32 gr./Kgr./80 min. of glucose; under the influence of the excess of insulin injected, glucose retention by the liver passes from a negative figure of −0,32 to a positive one of +1,70/Kgr./80 min. the total change on balance produced being, therefore, 2,02 gr./Kgr./80 min. Under the influence of the same excess of insulin, peripheral consumption is raised from 0,32 to 0,40 gr./Kgr./80 min. the excess of

[3] On the contrary, there seems to be rather an increase in the peripheral consumption of glucose after hepatectomy. See p. 51–52.

glucose taken up being 0,08 gr./Kgr./80 min. It is clear that doses of insulin higher than the physiologically secreted amounts of this hormone act almost exclusively on the liver, where they promote glucose-retention in a remarkable way, the ratio-hepatic action/peripheral action being 25 (table 2).

On the other hand, the smaller physiological doses of insulin exert a strong action on the peripheral tissues. This is shown by the results obtained on eviscerated and on pancreatectomized-hepatectomized animals receiving no insulin. The smallest figure we have obtained for the peripheral consumption of an animal completely deprived of insulin is 0,09 gr./Kgr./80 min. It will be remembered that this figure has been reduced to that for a normal blood-sugar level; in reality, a consumption of 0,24 gr./Kgr./80 min. has been observed at a mean glycaemia of 0,239 per cent.

By comparing this figure with that observed on the hepatectomized animal supplied with insulin from its own pancreas (0,32 gr./Kgr./80 min.), we find that physiological amounts of insulin promote the peripheral disappearance of 0,23 gr./Kg./80 min. of glucose. The figure obtained on the eviscerated animal (acutely pancreatectomized-hepatectomized) is an intermediate one (0,18

TABLE 2

AMOUNTS OF INSULIN AVAILABLE	AMOUNTS OF GLUCOSE TAKEN UP AT A NORMAL BS LEVEL GR./KGR./80 MIN.		RATIO: HEPATIC ACTION/PERIPHERAL ACTION
	Liver	Other tissues	
Super-maximal.....	+1,70	+0,40	
Physiological.....	−0,32	+0,32	
Difference.....	+2,02	+0,08	25

gr./Kgr./80 min.), showing that the insulin remaining in the tissues immediately after pancreatectomy still favours glucose consumption to a certain extent.

In order to determine the ratio: hepatic action/peripheral action of physiological amounts of insulin, we should know to what extent the insulin secreted by the pancreas inhibits sugar-formation in the liver of the fasting normal animal; in other words, we should know how much glucose would be poured into the bloodstream by the liver of an animal, completely deprived of insulin without the blood sugar level having changed. This figure cannot be obtained experimentally; even without taking into consideration the change in blood sugar level, the hepatic glucose output of a diabetic animal would probably vary considerably according to its state of nutrition and to the degree of ketogenesis attained. In the most favourable conditions, such as in the protein-fed diabetic animal, the liver supplies large amounts of glucose to the blood stream, covering the peripheral needs (approximately normal) and the renal excretion. Taking into account the inhibition of the hepatic output produced by the high blood-sugar level, we have estimated that the liver of a dog, kept at a normal blood sugar level and completely deprived of insulin would secrete into the bloodstream

approximately 0,85 gr./Kgr./80 min. (de Duve (27)). Although this figure rests on only very indirect evidence, it may serve to emphasize an important fact, for there is little chance that the hepatic action of physiological amounts of insulin is more than that of reducing the hepatic output from 0,85 to 0,32 gr./Kgr./80 min., i.e., that of favouring the retention of more than 0,53 gr./Kgr./80 min. of glucose. And we may take it that the ratio: hepatic action/peripheral action of these small doses of insulin will hardly exceed the value of 2 (table 3).

The same reasoning applied to the total action of a super-maximal dose of insulin furnishes for the ratio: hepatic action/peripheral action, a value of approximately 8 (table 4).

TABLE 3

AMOUNTS OF INSULIN AVAILABLE	AMOUNTS OF GLUCOSE TAKEN UP AT A NORMAL BS LEVEL GR./KGR./80 MIN.		RATIO: HEPATIC ACTION/PERIPHERAL ACTION
	Liver	Other tissues	
Physiological	−0,32	+0,32	
None	−0,85	+0,09	
Difference	+0,53	+0,23	2

TABLE 4

AMOUNTS OF INSULIN AVAILABLE	AMOUNTS OF GLUCOSE TAKEN UP AT A NORMAL BS LEVEL GR./KGR./80 MIN.		RATIO: HEPATIC ACTION/PERIPHERAL ACTION
	Liver	Other tissues	
Super-maximal	+1,70	+0,40	
None	−0,85	+0,09	
Difference	+2,55	+0,31	8

Finally, we may conclude:

1. That the higher the dose of insulin, the more it tends to act on the liver; the *maximal active dose* must therefore be much higher for the liver than for the other tissues.

2. That, whatever the dose of insulin present, and especially with the larger ones, much more glucose is taken up by the liver than by the other tissues under the influence of insulin; consequently the *maximal amount of glucose* which can be taken up under the influence of insulin at a normal blood-sugar level is also much larger for the liver than for all the other tissues.

Taken together, these facts prove that insulin exerts a direct and powerful action on the liver and that this action is far more important, quantitatively, than its peripheral action.

These results are in full agreement with those of Soskin's and, added to them, should afford convincing proof of the essential and central rôle played by the liver

in the processes of blood-sugar regulation. They raise many questions and lead to many conclusions, of which we can only, and very briefly, mention the most important ones.

One may wonder, first of all, how this important action has escaped the attention of so many authors and has even been emphatically denied by some. Two facts are mainly responsible for this: 1. As we have already mentioned above, neglect of the importance of the blood-sugar level has led to many erroneous results, particularly in those experiments where hypoglycaemia has not been avoided. It is clear that the antagonistic reactions occurring in hypoglycaemic animals consist essentially in a discharge of hormones which stimulate the glucose production of the liver and thereby electively oppose the hepatic action of insulin without interfering with its peripheral action. The use of impure insulin, contaminated by a blood-sugar raising factor, has also furnished faulty results. 2. The hepatic systems on which insulin acts are extremely fragile. We were able to note this fragility in a number of experiments (21) (23), in which we observed a deleterious effect on insulin action of various factors (adrenalin, anesthetics, hyperventilation, hypoventilation, spinalisation). Of all these factors, reduction of the oxygen-supply and spinalisation were the most noxious. The fact that insulin is inactive on the perfused liver is therefore not astonishing, especially if it be remembered that the liver is particularly well supplied in enzymes of all sorts and for that reason, very sensitive to autolytic influences. It may be further added that our experiments do not allow us to exclude the possibility that insulin, as has been suggested by Nielsen (52), must be activated by a substance of muscular origin, to be active on the liver.

If a complete survey of the earlier literature were made with these fact ins mind, it would be clear that a feeling of distrust toward the existence of a hepatic action of insulin was created not so much by lack of evidence as by the confusing amount of contradictory results obtained in this field. Once these contradictions are satisfactorily accounted for, the evidence in favour of the hepatic theory becomes overwhelming.

It may be objected to our experiments that the figures for peripheral glucose consumption obtained on anesthetized and artificially ventilated animals, submitted to severe operative interference, cannot be applied as such to the normal animal. This objection is easily met: 1. Our results are in close agreement with those obtained by various other authors who have carried out similar experiments, showing that the technique used in our experiments is not inferior to that used by other authors. 2. As far as the validity of the technique itself is concerned, an interesting fact emerges from a comparison of our results with those of Cherry and Crandall (10) and of Soskin, Essex, Herrick, and Mann (59). Cherry and Crandall (10) have measured the hepatic glucose output of normal unanesthetized dogs, by means of the angiostomy method; they find that the liver secretes 9,1 $\pm$ 0,68 mgr. of glucose p. 100 ml. of hepatic blood flow. According to the results of Blalock and Mason (1), the mean hepatic blood flow of the normal dog is 28,6 ml./Kgr./min. From these two figures it may be calculated that the mean hepatic glucose output of a normal unanesthetized dog, and consequently also

his mean peripheral glucose consumption, amounts to 0,21 gr. ± 0,02/Kgr./80 min. Using a similar method Soskin, Essex, Herrick and Mann (59) have measured simultaneously the hepatic glucose output and the hepatic bloodflow of anesthetized dogs, before and after administration of varying amounts of glucose. If we single out in their experiments the 6 figures obtained on dogs with a normal blood-sugar level, we find a mean value of 0,21 ± 0,12 gr./Kgr./80 min. There is no indication, therefore, that the anesthesia has greatly modified either the hepatic output or the peripheral consumption in these animals. Finally, we ourselves have found for the peripheral consumption of anesthetized, hepatectomized dogs a mean value of 0,32 ± 0,06 gr./Kgr./80 min. Neither the anesthesia nor the exclusion of the liver seem to have brought about any diminution of the capacity of the peripheral tissues to take up blood glucose. On the contrary, the figure found on the hepatectomized dog appears to be significantly higher than that observed on the intact animal. The obvious explanation of this difference seems to be that, in the hepatectomized animal, all the insulin secreted by the pancreas is carried out directly to the peripheral tissues, whereas in the intact animal, part of it is retained by the liver. This view is supported by the fact that, in the totally eviscerated dog, the peripheral consumption is much smaller, approaching the value observed on the intact animal (0,18 gr./Kgr./80 min.).

In the latter case no insulin at all is conveyed to the tissues during the experiment, but, considering the experiment is carried out immediately after the operation is performed, the effect of the insulin present in the tissues must be expected to wear off only gradually, which accounts for the fact that the glucose uptake of the eviscerated dog appears to be only slightly lower than the peripheral consumption of the intact animal.

3. Finally, it may be noted that both the hepatectomized and the eviscerated dog are perfectly sensitive to their own insulin as well as to externally provided insulin.

All these facts seem to deal satisfactorily with the objection that the important reduction of the capacity to utilize glucose under the influence of insulin, brought about by extirpation of the liver, could be due to a depressing influence of the operative interferences on the peripheral capacity of utilization. On the other hand, the various quantitative data by which we have tried to systematize our results may have to be revised in view of the fact that hepatectomy apparently induces a state of relative hyperinsulinemia in the peripheral tissues. If we take as a more probable value for the peripheral consumption of a normal dog, the figure of 0,21 gr./Kgr./80 min., observed on intact animals both by Cherry and Crandall and by Soskin *et al.*, the figures given above should be changed to the following values:

Hepatic retention with physiological amounts of insulin: −0,21
Peripheral consumption with physiological amounts of insulin: +0,21
Hepatic retention with complete lack of insulin: approximately −0,69.

In that case, the final figures given in tables 2, 3 and 4 should be replaced as follows (table 5).

It is clear that these corrections do not affect at all our main conclusions. In-

deed, they appear to provide corroborative evidence in favour of a prevalent action of insulin on the liver. From the comparison which has been drawn between our results and those of Cherry and Crandall and of Soskin et al., it appears that, through extirpation of the liver, the amount of glucose disappearing in the peripheral tissues under the action of physiologically secreted insulin is almost doubled. As the relation between the amount of glucose consumed under the influence of insulin and the amount of insulin present in the animal is represented by a typical saturation curve (Bouckaert, De Nayer and Krekels (4) (5)), it follows that the amount of insulin made available to the peripheral tissues by hepatectomy is more than doubled; in other words, that the liver normally retains the largest part of the insulin secreted by the pancreas. As has been shown, this fraction is not merely retained by the liver to be destroyed, but exerts a powerful action on this organ. The possibility remains, all the same, that the liver may also intervene in regulating the blood-sugar level, by allowing more or less insulin to reach the peripheral tissues.

e. *The final fate of glucose disappearing under insulin action.* A general answer to this question can easily be drawn from the results obtained in our laboratory

TABLE 5

TABLE	AMOUNTS OF INSULIN ACTIVE	GLUCOSE DISAPPEARING UNDER THE INFLUENCE OF INSULIN IN		RATIO: HEPATIC ACTION/PERIPHERAL ACTION
		Liver	Peripheral tissues	
II	Excess (maximal – physiological)	+1,91	+0,19	10
III	Physiological	+0,48	+0,12	4
IV	Total maximal dose	+2,39	+0,31	8

by Bouckaert and Stricker (3) in 1924 and by Lamers (38) in 1926. Bouckaert and Stricker have measured by direct calorimetry the heat production of rabbits receiving glucose and insulin and observe a maximum rise of 10 per cent. Lamers, using indirect calorimetry, has measured the total heat-production and the respiratory quotient of rabbits in the same conditions. According to his results, the basal metabolism suffers only slight variations but there occurs a definite rise in R.Q., approaching unity. From these data, the total amount of carbohydrate oxidized can be derived.

The experiments were performed before the method of compensation was standardized; in some the glucose was administered subcutaneously, in others intravenously but a precise measure of the quantity which had disappeared was not available. Luckily, in a number of those experiments, super-maximal doses of insulin were used and the glycaemic curve is contained within physiological limits; from the data obtained subsequently by Bouckaert, De Nayer and Krekels (4) (5), we know the exact amount of glucose which has disappeared under those conditions and can now compare this amount with the amount oxidized.

This comparison has been done recently in a paper by Bouckaert and De Nayer (1945) (7) and leads to the conclusion that only 50 per cent of the total

amount of glucose taken up in all the tissues has been oxidized. We must assume therefore that the other half has been stored under form of reserve substances, probably by oxidative synthesis. But, as the rise in glucose oxidation is not associated with a corresponding rise in total metabolism, it follows that an equivalent amount of protein and fat must have been spared as a result of the oxidation of the first half.

If we assume that a quantity 2 A has disappeared under insulin action, the final fate of this quantity may be gathered from the following data: A will be oxidized but the energy yielded by this reaction will be stored as chemical energy by the sparing of an equivalent amount of reserve substances; a further quantity α A will also be oxidized and the energy yielded will serve for the conversion of the remaining quantity $(1 - \alpha)$ A into reserve substances. Therefore, for every loss of glucose there is a corresponding gain of reserve substances, either spared or newly formed, and we may conclude that *when glucose disappears under insulin action the main result is the conservation and possibly the synthesis of reserve substances.*

A more detailed description of the processes involved raises four questions:

1. What are the reserve substances, which are spared or synthesized as a result of glucose disappearance under insulin action? The most important one is certainly glycogen. From the value of the R.Q. of the fasting animal, we deduce that part of its caloric requirements is furnished by direct oxidation of carbohydrate; this carbohydrate is almost exclusively present under the form of glycogen. When glucose is given along with insulin, this part of the caloric requirements is now met by the oxidation of glucose and an equivalent part of glycogen is spared. Moreover, many experiments show that glycogen is also newly formed when sufficient quantities of glucose and insulin are given. But the sparing action does not stop at glycogen and affects also fat and protein oxidation; otherwise a rise in R.Q. would not occur. And there is good evidence that, in certain conditions, fat and protein will not only be spared, but also be newly formed, when great amounts of glucose disappear under insulin action.

2. Do these results fit in with an essentially hepatic theory of insulin action? They definitely do so, for it will be remembered that the normal peripheral requirements are met mainly by glucose present in the blood. This glucose is provided, in the fasting animal, by formation from glycogen, protein and possibly fat, which takes place in the liver. Therefore the sparing action affects chiefly hepatic metabolism, where glycogenolysis and glyconeogenesis will be strongly inhibited, and it follows naturally that the main site of synthetic processes will also be the liver. Of course, peripheral metabolism will be influenced as well, especially by the smaller doses of insulin: this action will affect muscular glycogen reserves and peripheral stores of fat and protein.

3. Does the great amount of experimental work devoted to this subject support this theory? As we have pointed out before, many of the results obtained by different authors are contradictory, so that, taken together, they furnish insufficient evidence in favour of any theory of insulin action. It can be shown, however, that these contradictions are only apparent and are mainly due to differ-

ences of technique leading to erroneous interpretations. We can only deal very briefly with this important aspect of the problem, referring for further details to the work of de Duve (27), already mentioned, in which the former literature has been critically examined and reinterpreted.

It will be clear by now that the blood-sugar level plays the most important part in this reinterpretation. First of all, consideration of its importance allows the discarding of all experiments where marked hypoglycaemia has been provoked by insulin, insufficiently compensated with glucose, for the results obtained in those experiments are partly due to hormones antagonistic to insulin, such as adrenalin, diabetogenic pituitary factors, cortin, etc. This group will be found to comprise most of the experiments in which "diabetic" phenomena have been noted to occur after injection of insulin or in which the normal effects of insulin have failed to occur, for instance: those in which either no rise or a lowering of the R.Q. has been noted, those in which either no decrease or an increase in nitrogen excretion, or in ketogenesis and fat catabolism, have been observed, etc. Decreases in liver glycogen also chiefly occur in hypoglycaemic animals; this question has already been dealt with (see "The so-called glycogenolytic action of insulin", p. 42).

The question of blood-sugar level also intervenes in the interpretation of a second group of experiments, namely, those in which equal quantities of glucose are given to animals, one half of which are subjected additionally to an injection of insulin. Comparison of the results obtained on the insulinized animals with those observed on the non-insulinized animals frequently enforces the conclusion that *addition of insulin does not lead* to a greater formation of this or that substance than is produced by the glucose itself. Curiously enough, most authors have failed to note the important significance of this conclusion. If both the insulinized and the non-insulinized animal receive the same quantity of glucose, it is clear that in both animals the same quantity of glucose will have finally disappeared from the blood-stream into the tissues. If therefore a greater quantity of it is not found under one specific form, this only shows that *insulin does not favour one path of disappearance against the other, but treats all paths of disappearance alike.* The one great difference between the insulinized animal and the non-insulinized animal lies in the *difference in blood-sugar level* at which the disappearance of the same quantity of glucose takes place along the same paths. If the same experiment were performed in similar conditions of glycaemia, a greater quantity of glucose should have to be injected to the insulinized animal to maintain a blood-sugar level identical to that of the non-insulinized animal; the automatic influence of the glycaemic difference would then be eliminated and an increase in the formation of the substance studied with respect to the non-insulinized animal would indeed be noted.

Such results are extremely interesting, because they tend to show that *the fate of glucose disappearing under insulin action is not different from the fate of glucose disappearing under the automatic influence of an increase of its concentration in the blood, whatever the quantity of insulin available.* This fact is confirmed by the many experiments, in which the effects of glucose itself have been studied; it is

well known that provoked hyperglycaemia leads to an increase of the R.Q. and of hepatic and muscular glycogen reserves, exerts a sparing action on fat and protein catabolism and even furthers the synthesis of those substances. All these actions can be also attributed to insulin. Further confirmation is furnished by the experiments carried out on diabetic animals and showing that all the metabolic processes known to be furthered by insulin can be promoted without insulin being present, provided the blood-sugar level is sufficiently raised.

It becomes easily understandable hereafter why experiments performed on hypoglycaemic animals have furnished such misleading results. In a first stage of these experiments, insulin will promote the disappearance of the small quantities of glucose present in the blood-stream, creating hypoglycaemia; in a second stage, a stimulation of hepatic glycogenesis will take place under the influence of the blood-sugar raising factors secreted through reflex reactions against hypoglycaemia; glucose will then be secreted into the blood-stream and will be further metabolized under the influence of insulin in the tissues not involved in the antagonistic reactions, that is: mainly in the extra-hepatic tissues; the final result will be an elective furthering of peripheral consumption, frequently combined with activation of hepatic glycogenesis. The results might have been different if insulin had proved to be an activator of one specific reaction of glucose metabolism, such as glycogen synthesis, for instance, but we have seen that this is not the case.

Once these important facts are made clear, the interpretation of apparently conflicting results presents little difficulty and all experiments which are not open to similar criticism are found to agree with the theory expounded above. In a few cases, the problem is less simple and some results remain difficult to explain. Some of these are due, as has been shown above, to the presence of a glycogenolytic substance in certain samples of insulin. Another instance of them is furnished by the recent results of Britton and Corey (9) (12), in which it is stated that cortin, but not insulin, promotes the synthesis of hepatic glycogen. We have expounded elsewhere why we do not think their results with insulin to be conclusive (24) (27) and only wish here to emphasize the fact that cortin, as well as anterior pituitary extracts, further glycogen synthesis by furthering glyconeogenesis, and perhaps also by activating phosphorylase (Verzar), while insulin promotes glycogen formation in the liver by increasing the total amount of glucose taken up by the liver from the blood-stream. As a result of this, an increase in hepatic glycogen will be best obtained by the combined action of insulin and of cortin or anterior pituitary extract, for glycogen will be the only form under which the glucose taken up by the liver from the blood-stream as well as that produced in the liver from protein and fat can be stored. This has been conclusively proved by Marks and Young (47) and by Loubatières (42). But there is no reason why excessive stimulation of glyconeogenesis (by cortin) should not further glycogen formation either in the liver of pancreatectomized animals or in the perfused liver; on the other hand, inhibition of glyconeogenesis (by corticectomy) may alter the fate of glucose taken up by the liver under insulin action by favouring the paths leading to protein and fat against that leading

to glycogen; this will particularly be true if Verzar's theory be confirmed, as glycogen synthesis would then itself be impaired.

4. Finally one may wonder how insulin promotes glucose disappearance and creates such complex metabolic changes involving fat and protein as well as carbohydrate metabolism. To answer this question, it is necessary to outline briefly the theory of insulin action which will be further expounded at the end of this paper.

This theory is based essentially on the *analogy existing between the effects produced either by an increase in the blood-sugar level without addition of insulin or by a discharge of insulin without variation of the blood-sugar level.*

In both cases, as is shown by a comparison of the results of Bouckaert, De Nayer and Krekels (4) (5) with those of de Duve and Bouckaert (19), a surplus of glucose disappears from the blood-stream. In both cases, as may be inferred from our own results (20) compared with those obtained by Soskin, Allweiss and Cohn (57), Soskin, Essex, Herrick and Mann (59), the main site of this disappearance is the liver, only a relatively small fraction of the total amount disappearing in the extra-hepatic tissues. In both cases finally, as it has just been shown, the final fate of the glucose disappearing is the same.

The question stated above can therefore best be answered by identifying first the mechanism by which the concentration of glucose itself promotes its own disappearance. It will be remembered that this relationship is independent of all neuro-hormonal control and must be due to the biochemical properties of the tissues involved. The law of mass action, applied to the initial reaction by which glucose enters the metabolic processes, provides an adequate explanation of this phenomenon. Indeed, this initial reaction may be represented by the equation:

$$\text{Glucose } (+ \text{ A } \cdots) \rightarrow \text{X}$$

and its velocity will be increased by an increase in glucose concentration. But this will lead to an increase in the concentration of X, which in its turn will increase the velocity of its disappearance, and so on. By virtue of the law of mass-action, the increase in glucose concentration will finally accelerate the whole chain of metabolic reactions conditioning its disappearance. Moreover its final fate will be different in different tissues, according to the biochemical potentialities of each tissue. Muscle tissue, for instance, will mainly oxidise glucose and convert it into glycogen, fatty tissue will synthesize fat out of it, etc.

In addition to this, the sparing action of glucose on protein and fat must also be explained. A possible explanation may be furnished by the fact that glucose and its immediate derivates have greater reducing powers than most metabolic substances and are therefore more easily oxidizable.

In the presence of a limited supply of oxygen, the oxidation of glucose would, for that reason, be privileged against that of other substances, and the sparing action of glucose would be nothing but a consequence of the fact that, in most cases, fat and protein are oxidized in the cells only when oxygen is rendered available by relative scarcity of glucose. Such an interpretation, coupled with the notion of *"biochemical inertia"* emphasized by Soskin and Mirsky (58) will be found to afford a satisfactory explanation of the gradual shifting of metabolism

to protein and fat, brought about by inanition or low carbohydrate diets, and of the enhancing effect of rich carbohydrate diets on glucose tolerance. Variations of the hormonal balance are of course also involved in these processes; what we wish to point out is that they are not the only factor responsible for them.

If this interpretation of the automatic effect of glucose concentration on glucose consumption be accepted, it follows necessarily from the similarity between this effect and the action of insulin, that insulin must produce the same primary results as an increase of the blood sugar level. In other words, insulin must also accelerate the initial reaction by which glucose enters the metabolic processes and must therefore be an activator of the enzymes responsible for that reaction. Such an explanation would account for all the phenomena[4] which are to be attributed to insulin action. In particular, it would explain how *insulin allows glucose to exert its multiple effects on hepatic and peripheral metabolism at lower concentrations than would otherwise be necessary.*

This theory can be tested by examining whether the substance X of the equation is effectively formed in greater quantity under the influence of insulin; in other words, by determining the immediate fate of glucose disappearing under insulin action.

f. *The immediate fate of glucose disappearing under insulin action.*[5] We have intentionally represented by the symbol X the first substance into which glucose is converted in the tissues, because the nature of this substance is not known with certainty. According to one school, led by Parnas, it is glycogen; other authors, with Meyerhof, identify it with hexose-monophosphate. Both alternatives have been considered in our laboratory in connection with insulin action. In 1932, De Nayer (17) determined the amount of glycogen present in two homologous muscles of rabbits, whose n. ischiatici had been previously cut to prevent muscular contractions, one muscle being taken before, the other after an injection of insulin adequately compensated by a continuous infusion of glucose. He has found that, although super-maximal doses of insulin were given, there was no increase but also no decrease (sparing action) in muscle glycogen, as long as the

[4] But one. See later: i) The action of insulin on protein metabolism, p. 66.

[5] The authors wish to make it clear that when this paper was sent in for publication, the later American literature was not yet available in Belgium. Even though some of the facts and theories discussed in these later chapters may now appear obsolete in the light of the recent findings on glucose phosphorylation by the Coris and their co-workers, they have preferred not to alter the original text in view of the material difficulties it would involve, and to go on stating the case as they were able to make it out under the conditions of enforced isolation imposed by the war.

Mention must at least be made of the recent announcement made by Price, Colowick and Cori (J. Biol. Chem. **160**: 633, 1945) that insulin is capable of reactivating "in vivo" and "in vitro" muscle hexokinase previously inhibited by anterior pituitary extract. Although this important observation affords only a partial explanation of the action of insulin, it constitutes the first definite step towards the elucidation of its biochemical mechanism. It also confirms the conclusion drawn from our own results that insulin must act as an activator of hexokinase. As to the nature of this activation and of the part played by hexokinase, the new evidence brings nothing either to prove or disprove the tentative explanation put forward in this paper.

blood-sugar level remained normal. Only by increasing the amount of glucose given and creating hyperglycaemia, did he succeed in promoting further glycogen synthesis in the muscles. Here again, the importance of the blood sugar level and the superiority of the method of compensation show quite clearly, when the results obtained by De Nayer are contrasted with those achieved by other authors in similar experiments.

Lately, de Duve and Hers (22) (25) have performed almost identical experiments, this time determining the amount of hexose-monophosphate. In the latter experiments, further precautions were taken and a control set of determinations was made on animals suffering the same treatment, but receiving an infusion of isotonic NaCl instead of glucose and insulin. A definite rise in the content of muscle hexose-monophosphate was observed in the insulinized animals, corresponding to a major part of the glucose taken up by the periphery.

This experiment, compared with that by De Nayer, may be termed crucial, both in regard to glucose metabolism and to the problem of insulin action. It shows that the substance X is hexose-monosphosphate, at least for the muscles, thereby confirming Meyerhof's theory, and it proves that insulin promotes the reaction leading from glucose to hexose-monphosphate, thereby confirming our theory of insulin action. The identity between the action of insulin and the automatic effect of hyperglycaemia is further demonstrated by comparing these results with those recently obtained by Leipert and Kellersmann (40), who have shown that infusion of excess glucose leads to an increase of muscle hexose-monophosphate, without affecting its content in glycogen.

We have not effected a similar test on the liver, the reason being that comparative determinations cannot be made on the liver of the same animal, without disturbing the glycaemic balance. But there are good reasons to believe that the uptake of glucose by the liver also occurs through phosphorylation. We know, for instance, that adrenalin strongly antagonizes the action of insulin and it has been shown that adrenalin acts on phosphorylative processes (see p. 68). According to Leipert and Kellersmann (40), absorption of glucose increases the content in esterified phosphorus of the liver; there is a good chance, consequently, that insulin does the same. Colowick, Kalckar and Cori (1941) (11) have shown glucose phosphorylation to take place in hepatic extract and have made a detailed study of this reaction (see p. 60). Finally, recent experiments by Kaplan and Greenberg (1944) (33) (34) (35) show that glucose tolerance is directly related to the amount of adenosine-triphosphate present in the liver and that both glucose and insulin increase this amount. As will be seen, adenosine-triphosphate plays the leading part in glucose phosphorylation.

It can reasonably be assumed, therefore, that the immediate fate of glucose disappearing under insulin action is to be converted into hexose-monophosphate, in the liver as well as in the muscles and other tissues.

g. *General theory of insulin action.* It may be useful, at this stage, to regroup the various facts discussed until now and to show briefly how they serve to support the theory of insulin action put forward in this paper. This section may be considered as a summary of the previous ones.

I. The most important fact which must be kept in mind is the important part played in blood-sugar regulatory processes by the blood-sugar level itself. The blood-sugar level regulates glucose consumption and production in the tissues both directly and indirectly.

1. By virtue of the law of mass action, the velocity of the reactions which condition glucose consumption is increased by a rise and decreased by a fall in blood-sugar level. Consequently, blood-sugar consumption is directly related to the blood-sugar level, independently of all neuro-hormonal processes, and this relationship has been shown to be linear within a range of glycaemia not too far removed from its normal value.

2. Hyperglycaemia provokes blood-sugar lowering neuro-hormonal reactions; hypoglycaemia stimulates blood-sugar raising neuro-hormonal factors.

II. Application of these principles to experiments concerned with the action of insulin leads to the important conclusion that variations in the blood-sugar level may considerably affect experimental results:

1. Hyperglycaemia may simulate the action of insulin, by automatically promoting glucose disappearance.

2. Hypoglycaemia will strongly antagonize the action of insulin, not only by the resulting automatic decrease in glucose consumption, but also by stimulation of antagonistic factors such as the orthosympathic system, adrenalin, cortin, anterior pituitary hormones, etc.

Consequently, all experiments where marked hypoglycaemia occurs should be discarded, as the results furnished by them are compound actions of insulin and of antagonistic factors. Moreover, in all experiments which are not liable to such criticism, the automatic influence of blood-sugar variations must be taken into account, either by using a method in which all the animals are maintained at the same, preferably normal, blood-sugar level by a continuous infusion of glucose, or by reducing mathematically the results obtained to the same glycaemic level. Both these methods have been described and extensively used by us.

III. As a further precaution, the brand of insulin used in the experiment must be tested for the presence of a blood-sugar raising impurity, endowed with strong glycogenolytic properties. As we have been able to show, the use of such like insulin may lead to gross experimental errors.

IV. By using a method in which all these conditions are fulfilled, we have been able to demonstrate conclusively the existence and the primary importance of the hepatic action of insulin. Whatever the quantity of insulin administered, considerably more glucose disappears in the liver than in the other tissues under its influence. An excess of insulin which does not further promote peripheral glucose consumption, is still capable of furthering glucose retention by the liver.

V. The great number of conflicting results obtained with insulin is mainly due to neglect of the blood-sugar level. Proper reinterpretation of those experiments eliminates most discrepancies and allows the fate of glucose disappearing under insulin action to be accounted for satisfactorily. It becomes quite clear that, of the actions investigated, insulin produces no metabolic changes other than those

produced by the glucose itself: a rise in R.Q. occurs, associated with a negligible rise in total metabolism, thereby showing that if more glucose is oxidized, it is only utilized instead of other substances, glycogen already stored, fat and protein; when great amounts of glucose disappear, this sparing action is prolonged by new formation of glycogen, fat and protein. On the whole, when an excess of glucose disappears under insulin action, this glucose is stored as equivalent glycogen, fat and protein, either spared or newly formed. The real action of insulin is to allow glucose to exert similar actions at lower blood-sugar levels than would otherwise be necessary.

VI. The proximate fate of glucose disappearing in the tissues, whether under the action of insulin or not, is conversion into hexose-monophosphate.

VII. There is a strong analogy between the effects of an injection of insulin associated with a normal blood-sugar level and those of an increase in the blood-sugar level without change in the insulin supply. In both cases, an excess of glucose disappears, mainly in the liver, secondarily in the muscles and other tissues, is converted into hexose-monophosphate, which, in its turn, is partly oxidized, thereby sparing an equivalent quantity of glycogen, fat and protein, partly converted by oxidative synthesis, into glycogen, fat and, even to a certain extent, protein.

VIII. The effects produced by an increase in the concentration of glucose can only be explained through the fact that, by virtue of the law of mass-action, more glucose is converted into hexose-monophosphate. All the metabolic changes observed may be considered as necessary consequences of this primary change, the two main factors involved being a successive furthering of the whole chain of metabolic reactions concerned in glucose metabolism (mass effect), and the privileged oxidation of hexose-monophosphate and its derivatives, as compared with less oxidizable substances, such as fat and protein.

IX. We are finally led to conclude that insulin must act on the same primary reaction and in the same way as an increase in the concentration of glucose, and must therefore increase the velocity of this reaction. Insulin thereby appears as an activator of the enzymatic system concerned with glucose phosphorylation in the tissues, and this action of insulin is in itself sufficient to account for all the metabolic changes which appear to be a consequence of its influence (except one: see later i, p. 66).

h. *The biochemical mechanism of insulin action.* So far, we have been able to trace down the biochemical point of attack of insulin to the enzymatic system responsible for glucose phosphorylation in the tissues. If we want to probe further into the intimate mechanism of insulin action, our first concern must be to gather more particulars about the mechanism of glucose phosphorylation itself on one side, and about the biochemical properties of insulin on the other side, and to see whether and how both can be reconciled satisfactorily.

Luckily, the phosphorylation of glucose, together with other important phosphocatalytic processes, has been extensively studied, especially in later years. It was first seen to take place in yeast cells, but its intervention in the metabolism of mammalian tissues was long in doubt; more recently, however, the occurrence

of direct esterification of glucose into hexose-phosphate has been observed by a number of authors on a great variety of animal tissues and even in cell-free extracts of liver, brain, and heart-tissue; it has been subjected to a close study particularly by the Coris and their co-workers (Colowick, Kalckar and Cori (1941) (11)).

The findings made on yeast-cells and on animal tissues furnish closely similar results and enable a provisional picture of this reaction to be drawn.

There is no doubt that the main reaction consists in an exchange of phosphate between adenosine-triphosphate (ATP) and glucose, through which glucose is converted into hexose-monophosphate, ATP into adenosine-diphosphate or adenylic acid. But the complete process is more complex.

Already the early experiments of Meyerhof on yeast-metabolism have shown that yeast-extract loses very quickly the capacity of utilizing glucose, although it shows no loss in its power to metabolize starch or glycogen. Similar findings have been made on mammalian tissues. Isolated muscle or liver tissue becomes rapidly inactive towards glucose, while retaining its capacity to break down glycogen; muscle-extract, though endowed with unimpaired glycogenolytic properties, does not in the least attack glucose; and it is only lately that it has become possible to prepare cell-free extracts of certain organs, capable of phosphorylating glucose "in vitro."

All these findings tend to show that glucose cannot be phosphorylated as such, but must first be brought into an active state, before becoming vulnerable to the phosphorylating system; they also seem to indicate that the systems responsible for this activation are of a very fragile nature. This assumption has been confirmed by Meyerhof, who has isolated from yeast-cells a system capable of reactivating inactive yeast-extract and to which he has given the name "*hexokinase;*" a similar system has been shown to exist in fresh muscle-extract by Ahlgren, who has christened it "*glycomutin;*" an analogue of hexokinase has been isolated lately from brain-tissue by Ochoa (53). Nevertheless, no explanation has been yet proffered as to the nature of this "activation", which the molecule of glucose is supposed to undergo.

The following hypothesis, based on the reactions conditioning glycogen breakdown in the muscles, has been put forward by one of us (27) to account for it: It will be remembered that the first reaction occurring in glycogen breakdown is a fixation of inorganic phosphate on glycogen (phosphorolysis), giving birth to glucopyranose—I. phosphate (Cori-ester). Hexose-monophosphate is further transformed into hexose-diphosphate by receiving one phosphate group from ATP (phosphorylation). This second reaction is very similar to the one through which glucose is phosphorylated, and it is to be noted that Cori-ester, which has the same structure as blood-glucopyranose, is no more able than glucose to react as such with ATP. It must previously be converted into fructofuranose-6-phosphate (Neuberg-ester). We may therefore, with Parnas (55), define the conditions of vulnerability of hexose-monophosphate to ATP as the following: the negative group must be attached to carbon 6 and carbon 1 must be set free under the form of a primary alcohol function by a changing of the pyranic into a

furanic cycle. It may be reasonably assumed that similar changes will have to take place inside the molecule of glucopyranose, prior to its phosphorylation by ATP; in that case, active glucose would prove to be fructofuranose, the action of hexokinase or glycomutin would be a temporary fixation of glucose by its carbon 6 associated to a regrouping of its pyranic cycle into a furanic one, and the first product of glucose phosphorylation would be fructo-furanose-1-phosphate (Robison-Tanko ester). It is to be noted that this ester has recently been isolated from the liver of rabbits by Pany (54). This hypothesis would explain also why fructose is more easily phosphorylated than glucose; and would account for the fact that the presence of an active form of glucose in the blood has been inferred mainly from experiments showing that the optical activity of blood glucose was inferior to the value calculated from its reducing power.

Activation of glucose is not the only condition necessary to render glucose phosphorylation possible; all the authors who have studied this reaction agree on the necessity for simultaneous oxidative processes, acting on a variety of substrates, amongst which pyruvic acid seems to be the most prominent one. Cozymase appears to play an important part in this oxidation. One may wonder with what particular stage of the reaction the oxidation of pyruvic acid is coupled. It is very improbable that it would be phosphorylation itself, as splitting of ATP is highly exothermic in itself. Two alternatives remain, which have been advocated:

According to Kaplan and Greenberg (1944) (33) (34) (35), the energy supplied by the oxidative process is used for the building up of ATP. The rate of phosphorylation of glucose being directly related to the amount of adenosine-phosphates available, it would be indirectly dependent on the intensity of the oxidations conditioning their synthesis. If we accept this theory we must assume that glucose can also be oxidized without being phosphorylated and that this oxidation can catalyse the synthesis of ATP, which in its turn will promote glucose phosphorylation. Otherwise, the increase in liver ATP observed by the authors after administration of glucose cannot be explained. Indeed, the experiments by Colowick, Kalckar and Cori (11) seem to support this assumption, for they start with free adenylic acid; the primary process in these experiments must necessarily be the building up of ATP.

On the other hand, there is little to show that similar processes occur "in vivo," where ATP synthesis seems to be correlated with the breakdown of hexose-phosphate rather than of glucose and can occur, as has been clearly pointed out by Needham (49), at two different stages of this breakdown. As is well known, adenylic acid by reacting directly with phosphopyruvic acid, is rephosphorylated and regenerates ATP; but the oxidative formation of phosphopyruvic acid itself, or rather of phosphoglyceric acid, from triose-phosphate, is also coupled with resynthesis of ATP, from ADP and free phosphate this time. The recent findings of Warburg and Christian (63) and of Negelein and Brömel (50) (51), explain this coupling, showing that phosphoglyceraldehyde takes up one more phosphate group and that the diphosphoglyceric acid, formed by oxidation of diphosphoglyceraldehyd, subsequently abandons one phosphate group to ADP, converting it to ATP.

An interesting fact emerges from these considerations, namely, that hexose-diphosphate breakdown can catalyse the synthesis of more adenosine-phosphate than is consumed by its own formation out of glucose. The results of Kaplan and Greenberg can therefore be as easily explained by assuming that synthesis of ATP is not prior to glucose phosphorylation but is a consequence of it, through being promoted by the further breakdown of hexose-phosphate. The latter explanation must indeed be true for the muscles, for it has been shown by Leipert and Kellersman (1941) (40), that the accumulation of hexose-monophosphate occurring in the muscles after absorption of glucose goes together with a corresponding loss of ATP. The fact that, in the liver, hexose-phosphate and ATP both increase, might be considered as evidence in favour of Kaplan and Greenberg's theory but it could also be interpreted as a sign that hexose-phosphate is much more rapidly consumed in the liver, which is indeed the case. For we know that glucose is much more rapidly utilized by the liver than by the muscles. Even so, it is not possible to discriminate between both theories, for the advocates of the former might argue that this superiority of the liver is precisely due to the fact that liver tissue possesses more ways of building up ATP than muscle tissue.

If we accept the second alternative, that ATP synthesis is only promoted as a result of hexose-phosphate breakdown, we must find another explanation of the coupling of glucose phosphorylation with pyruvic acid oxidation. This can satisfactorily be done by relating the oxidative process to the preliminary activation of glucose. Activation of a molecule consists in the bringing of this molecule to a higher energetic level and must necessarily be endothermic; a coupling of the kind we surmise appears therefore as a necessity. Moreover, if it be assumed that hexokinase or glycomutin work in conjunction with oxidizing systems, their natural fragility is easily explained.

In short, our actual knowledge concerning glucose phosphorylation allows us to distinguish in it 4 different steps:

1. Adsorption of glucose on glycomutin.
2. Activation of the adsorbed glucose by glycomutin, possibly by conversion into fructo-furanose.
3. Phosphorylation of the activated glucose by ATP.
4. Oxidation of various carbohydrate derivates, principally of pyruvic acid, coupled either with ATP synthesis or with glucose activation.

It is remarkable that an action of insulin on each of these four stages has been described.

Let us consider first the action of insulin on the last of these steps. Early experiments by Polonovski and his co-workers have shown a lowering by insulin of the total amount of intermediate carbohydrate derivates present in the blood. In 1940, activation by insulin of pyruvic acid disappearance was observed simultaneously by von Euler and Högberg (28) and, in our laboratory, by Delrue and De Keyser (15). Various biochemical experiments have also furnished corroborative evidence of an intervention of insulin in the oxidation of carbohydrate by-products. On the other hand, insulin has long been suspected of being an activator of specific cellular oxidation, from the fact that its potency is dependent on the number of disulphide bonds present in its molecule. It is well known that

reduced insulin is totally inactive. A parallel was quickly drawn with glutathione and the natural inference was that insulin could act as hydrogen carrier in cellular oxidations.

These various facts appeared to fit in perfectly with the classical theory, in which the essential action of insulin is described as a furthering of glucose oxidation, and they even supplied a plausible explanation of this action.

According to this "*metabolic theory*," which has been advocated by Polonovski and by many other authors, the main action of insulin consists in an activation of intermediate carbohydrate oxidations and the intensified disappearance of glucose is a consequence of the metabolic "vacuum" thus created.

There are two objections to a theory of that kind. In the first place, even though there is no doubt that insulin has a furthering effect on the oxidation of intermediate products of carbohydrate metabolism, it is clear that this general activity must be related to a highly specific object. Insulin can not be replaced by any other substance, though there are many which are known to activate intermediate oxidations. Glutathione, for instance, does not act the same way as insulin; on the contrary it seems to inhibit insulin action (Levine *et al.* (1939)) (41), possibly because its disulphide-groups take the place of those of insulin and further similar processes without, however, co-ordinating them with a specific system, as insulin does. It must be remembered also that integrity of the disulphide-groups is not the only condition of insulin activity. We must conclude therefore that the action on intermediate metabolism is only one aspect of insulin action; though it is essential in many ways, it is not specific in itself but appears rather like a means through which insulin exerts its specific activity.

In the second place, if we assume the furthering action on oxidations to be the primary action of insulin, then we must admit that insulin promotes blood-sugar disappearance by creating a negative gradient of glucose. But, in that case, there should be a decrease not an increase in hexose-phosphate after insulin and the similarity between the effects of insulin and those of hyperglycaemia becomes difficult to explain. This similarity as well as other experiments which will be mentioned below show that it is the other way about and that insulin creates a positive gradient of glucose. When insulin is given, glucose does not disappear more rapidly because room is made for it, but because more can be stuffed into the same room. Of course during prolonged action, further accumulation of the primary products of glucose absorption will be facilitated by the decongestioning effect of the metabolic action.

Now that we know that oxidation of byproducts of carbohydrate metabolism is a necessary adjunct of glucose phosphorylation, all these difficulties vanish and the specific action of insulin is readily identified. What characterizes insulin and differentiates it from other unspecific activators is that it has the additional property of directing the energy it helps to set free, towards a strictly specific end: glucose phosphorylation. Whether it does so by furthering ATP synthesis or by promoting glucose activation remains to be seen.

To discriminate between these two alternatives, we can apply the same reasoning as before. If the primary action of insulin is to promote oxidative synthesis

of ATP, then it must further glucose absorption by promoting indirectly the phosphorylative disappearance of activated glucose and thus making extra room for more glucose to occupy. On the other hand, if insulin promotes the activation of glucose itself, then it must increase the amount of activated glucose present in the tissues. Experimental evidence seems more in favour of the latter alternative.

It will be remembered that quite a number of early experimental results have been alleged in support of a theory commonly known as the "*permeability theory.*" According to this theory, which rests mainly on results obtained by Loewi and other authors on blood-cells and blood-vessel tissue, the action of insulin consists essentially in a furthering of the adsorption of glucose on the surface of the enzymatic structure responsible for its ulterior metabolism. In another theory also advocated by some authors on the faith of experimental evidence, insulin is said to favour the conversion of ordinary, stable dextro-rotatory glucose into a labile, more levo-rotatory form; in other words, to promote glucose activation. It is true that both these theories have been severely criticized and that the evidence on which they are based has been shown to be inconclusive. It must be noted however that opposite changes have never been asserted to occur and that later experimental results have since been added to their credit.

For instance, a series of experiments by Soula and his co-workers show that, when glucose is administrated, part of it is stored, *as glucose*, in the peripheral tissues, and is subsequently returned to the blood-stream; insulin promotes this storage. According to the authors this storage takes place in the intercellular fluid contained in the lacunar spaces (stockage lacunaire); but this phenomenon may be due as well to a reversible process of adsorption-activation. More recent experiments by Lundsgaard (45), however, do not confirm this storage, but they show that the acceleration of the transfer of glucose from the blood into the muscles cannot be due to a lowering of the glucose concentration inside the muscle-cell, and must therefore be due to an active surface process. The extensive work carried out by Thomas (61) (62) on the rotatory power of blood sugar must also be mentioned in support of the activation theory.

Consequently, the only theory which seems to reconcile all experimental results satisfactorily, is the one in which insulin is considered as an activator of the coupled processes of glucose activation and intermediate carbohydrate derivates oxidation. Indeed, if our first conclusion be accepted that insulin must necessarily act on the same reaction as does the blood-sugar level itself, in other terms on the initial reaction of glucose metabolism:

$$\text{Glucose} \rightarrow \text{X}$$

then we must necessarily accept the biochemical interpretation propounded, which is nothing but the logical inference of the first, as X turns out to be nothing else but activated glucose.

We may therefore conclude that the main action of insulin appears to be an activation of a complex enzymatic system, for which we may retain the name "glycomutin." This system fixates blood glucopyranose and converts it to a

labile, less dextro-rotatory form, possibly fructo-furanose; it borrows the energy necessary to this conversion from the oxidation of various carbohydrate derivates, particularly of pyruvic acid. The activated glucose subsequently reacts with adenosine-triphosphate and is transformed into hexose-monophosphate.

The hexose-monophosphate formed may be either polymerised into glycogen or broken down into pyruvic acid. This breakdown is facilitated by the rupture of the chemical balance between hexose-monophosphate and pyruvic acid, as the former's concentration increases while the latter's initially decreases. Moreover, hexose-monophosphate breakdown is itself coupled with ATP synthesis. It follows that the amount of glucose which will be absorbed depends on the velocity of the subsequent breakdown of hexose-monophosphate, which favours it both by removing hexose-phosphate and by regenerating ATP. This breakdown appears to be much slower in the muscles than in the liver. Finally, the pyruvic acid produced from hexose-phosphate will be mainly oxidized, this process involving a corresponding decrease in fat and protein breakdown, but will also be partly used, when an excess of glucose is absorbed, for the synthesis of fat and possibly protein. It will be noticed that insulin, by acting as it does, simultaneously favours the removal of glucose from the blood-stream and the privileged oxidation of its final derivates, while the maximum velocity of glucose absorption under its influence depends essentially on the velocity with which hexose-monophosphate can be converted into those derivates through a chain of reactions which is not, apparently, influenced by insulin.

It is possible that direct oxidation of activated glucose as well as oxidation of other derivates may also play a part in the synthesis of ATP. In that case, a furthering action of insulin on these reactions might possibly also occur.

i. *The action of insulin on protein metabolism.* As has been shown above, all the actions of insulin appear to be satisfactorily interpreted by the theory just described. There exists however one action of insulin which seems to be independent of the others; it is an inhibition of protein hydrolysis, which has been demonstrated by the experiments of Lacquet, De Nayer and Bouckaert (36) (37).

Comparison of their results with those published more recently by Mirsky (1938) (48) enforces a conclusion somewhat different from that originally drawn by the authors. In a first set of experiments, carried out on normal and on pancreatectomized dogs, they have confirmed the fact that the concentration of amino-acids in the blood is raised by pancreatectomy and decreased by insulin. In a second set of experiments, they have compared the blood-amino acid curve of dogs treated with insulin with that of untreated animals, during continuous infusion of various amounts of glycine, and have observed that the mean blood-amino acid level was lowered by insulin in the animals receiving small amounts of glycine, but was raised by insulin in the animals receiving moderately large and large amounts of this amino-acid.

These results tended to show that, as long as the concentration of amino-acids in the blood was sufficiently low to allow a certain degree of proteolysis to proceed in the tissues, addition of insulin inhibited this process, but that, when the blood amino-acid level became high enough to reverse the process and induce proteo-

synthesis in the tissues, the latter process was likewise inhibited by insulin. In other words, insulin appeared to inhibit both the hydrolysis and the synthesis of proteins.

Bouckaert, De Nayer and Cassiman (1934) (6) subsequently showed that, even in the animals receiving small amounts of glycine, the blood amino-acid level was raised by insulin, when the blood-sugar level was maintained by an additional infusion of glucose. These results served to emphasize a second action of insulin on protein metabolism, really dependent on the disappearance of glucose, namely, a strong inhibition of deamination in the liver; as a result of this second action, the removal of amino-acids from the blood-stream is slowed down to such an extent that their concentration in the blood increases despite the simultaneous inhibition of peripheral proteolysis.

The results obtained by Mirsky (1938) (48) confirm the existence of two independent actions of insulin on protein metabolism, one of them being an inhibition of deamination in the liver, the other being an inhibition of proteclysis in the peripheral tissues. But some of the results he has obtained on eviscerated animals are in complete disagreement with the conclusion drawn by Lacquet and his co-workers that insulin inhibits proteo-synthesis as well, for they show that insulin actually promotes the removal of amino-acids from the blood-stream into the peripheral tissues. If we assume that insulin exerts a similar action on the peripheral tissues of the intact animal, another explanation must be found for the fact that the concentration of amino-acids in the blood of the insulinized animal is nevertheless higher than in the non-insulinized animal, when large amounts of glycine are provided. One explanation appears to be that the infusion of additional quantities of glycine affects the hepatic metabolism of the insulinized animal in more or less the same way as the infusion of additional quantities of glucose, and enhances the inhibitory effect of insulin on hepatic deamination. It could be that only a small part of the glycine provided is deaminated and that the furthering by insulin of the oxidation of its non-protein products is sufficient to account for a subsequent inhibition of the deamination of the remaining part. Another possible explanation is that insulin inhibits the removal of amino-acids from the blood-stream by the kidneys, for Mirsky's experiments are carried out on nephrectomized animals.

Whatever the explanation may be, Mirsky's results appear to be quite conclusive and it must be accepted that insulin not only inhibits protein breakdown in the peripheral tissues but promotes the building up of protein from amino-acids as well. This view is strongly substantiated by many recent experiments carried out by Young, Gaebler and other authors, in which it is shown that the sparing and building up of protein, which occurs under the influence of anterior pituitary extracts, requires the presence of sufficient amounts of insulin.

It is to be noted that the inhibition of hepatic deamination by insulin appears to be due largely to the disappearance of extra glucose in the liver, whereas the furthering of protein synthesis in the peripheral tissues seems to be the result of a direct action of the pancreatic hormone on these tissues. The former action may, therefore, be taken as a consequence of the general action of insulin on

glucose metabolism described in the preceding section; the latter appears to be entirely independent of it.

j. *The antagonism between insulin and adrenalin.* It has been shown by De Nayer (18) that adequate quantities of adrenalin may inhibit completely the hypoglycaemic effect of even strong doses of insulin. More recently, de Duve, Marin and Bouckaert (23) have observed that addition of very small doses of adrenalin to the glucose supplied by continuous infusion to insulinized animals leads to a considerable decrease of the quantity of glucose needed to maintain the blood-sugar level normal. In the latter paper, an attempt has been made to reconcile the recently gained information concerning the biochemical mechanism of adrenalin action with that obtained on insulin, in a way which would account for the antagonism between the two hormones. Two facts seem to be of importance:

1. It is remarkable that both insulin and adrenalin increase the amount of hexose-monophosphate present in the tissues. It is not unusual to have two antagonistic factors acting synergically on the formation of one particular substance; the same occurs with the synthesis of hepatic glycogen which is favoured both by insulin and by anterior pituitary extracts or cortin. Indeed part of their antagonism may be precisely due to this apparent synergy, for both the origin of the substance formed and the mechanism of its formation are different when the effect is provoked by insulin or by its antagonist. In the case of hepatic glycogen, we have seen that insulin favours its formation from glucose, by promoting glucose phosphorylation, while anterior pituitary extracts and cortin further its synthesis from protein and possibly fat, by activating glyconeogenesis. In the case of hexose-phosphate, insulin promotes its formation by facilitating the phosphorylation of glucose by ATP, while adrenalin does the same, by activating phosphorolysis of glycogen at the expense of inorganic phosphate. It is evident that activation of one of these mechanisms will necessarily tend to counteract the other.

2. Another important point is that phosphorylation is coupled with oxidative reactions, while phosphorolysis is favoured by reducing processes. Now, we know that insulin also favours the coupled oxidations, particularly that of pyruvic acid. On the other hand, it is well known that adrenalin furthers the formation of lactic acid, which is the product of pyruvic acid reduction; moreover, after a detailed study of the action of adrenalin on glycogen-phosphorolysis, Lee and Richter (39) come to the conclusion that only two hypotheses could account for its mechanism, one of which being that adrenalin furthers the coupling between reducing substances and phosphorylase. We have there a second remarkable instance of the antagonism between the two hormones and a common substrate of their conflicting influences could be cozymase which, in the reduced state, reduces pyruvic acid into lactic acid, and in the oxidized state, intervenes in pyruvic acid oxidation. Recent results by Göbell (31) (32) seem to confirm this assumption, for they show that administration of nicotinamide, one of the main constituents of cozymase, enhances the actions both of insulin and adrenalin.

Conclusion. Except for its independent action on protein synthesis, all the known effects of insulin, as well as the great majority of experimental results obtained on insulin, can be satisfactorily explained by the theory of insulin action put forward in this paper. This theory has been elaborately described (see sections g) and h) and need not be repeated here.

The main points we wish to emphasize are the importance of the blood sugar level, the importance of the hepatic action of insulin, the close similarity between the effects of insulin and those of an increase in blood-sugar level, and the importance of phosphocatalytic metabolism in the metabolism of glucose.

From these various facts, the following picture can be drawn of the part played by insulin in blood-sugar regulation.

The fasting blood-sugar level is the result of an equilibrium between glucose production and glucose consumption. If we assume all neuro-hormonal influences to be stabilized and thus to act in a constant manner, this equilibrium will be essentially a chemical equilibrium, and the resulting blood-sugar level will be the concentration consistent with this equilibrium. The state of equilibrium will be attained when the quantity excreted by the liver will become equal to the quantity consumed by the other tissues. Such a state can always be attained and will automatically be attained by a readjustment of the blood-sugar level, for we know that the total quantity of blood-glucose either consumed or produced is a direct function of the blood-sugar level.

Insulin has the power of modifying this function so that a progressive variation of the amount of insulin present in the tissues will create an infinite number of states of equilibrium, each being characterized by a particular concentration of glucose.

Therefore the basal function of the pancreatic islets will be to maintain a constant level of insulin in the tissues, corresponding to that particular state of equilibrium characterized by a physiological value for the blood-sugar level.

The main antagonists of insulin in this respect are the anterior pituitary hormones and cortin, which act mainly by increasing the production of glucose in the liver, by inhibiting peripheral oxidation of glucose and even by directly opposing the action of insulin.

The second, intermittent action of insulin is to accelerate the return to a normal blood-sugar level whenever hyperglycaemia has occurred, whether through digestive absorption or for any other reason. In that case, an excess of insulin is provided by the pancreas and creates temporarily a new state of equilibrium, characterized by a hypoglycaemic concentration of glucose. The return to a normal blood sugar level will then be accelerated but may be followed by hypoglycaemia. This will be dealt with by a discharge of adrenalin which will reverse the process, and so on.

The main antagonist of insulin, in this urgency function, is, of course, adrenalin.

SUMMARY

In this paper the whole bulk of experimental work carried out on the problem of insulin action at the physiological laboratory of the university of Louvain has

been summarized and discussed in connection with recent data obtained elsewhere. The main points, which will be found summarized under the heading "General theory of insulin action" (p. 58), lead to the conclusion that insulin acts on the chain of reactions which leads, in the liver as well as in other tissues, from blood-glucose to tissue hexose-monophosphate.

The next section is devoted to a close discussion of the biochemical mechanism of this action and it is shown that the only explanation which seems compatible with all the actually known facts is that insulin facilitates simultaneously the adsorption of glucose to a specific enzymatic system responsible for the conversion of glucose into an active, labile form, which is subsequently phosphorylated by ATP, and for the oxidation of various carbohydrate derivates, particularly of pyruvic acid, this process being coupled with the former.

Some aspects of the action of insulin on protein metabolism and of the antagonism between insulin and adrenalin are finally discussed.

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OPTIMAL GROWTH OF THE RAT[1]

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The importance of the rat in studies of growth and nutrition has been universally recognized yet the data on growth and physiological performance have not been reviewed comprehensively since 1924, the date of Donaldson's (3) revised classical treatise. The need for correlation of old and new data is emphasized by the statement of Donaldson (1924, p. x) that the performance of his rats, although a reasonable standard for that period, should not be considered the ultimate criterion. He deduced that "these rats show the characters commonly found in (this and) other laboratory colonies, but they represent neither the best animals nor those in the ideal condition, nor do they necessarily yield the values which will be found in Albinos ten years hence." Abundant evidence in confirmation of this assertion has been provided by the discovery, during the past two decades, of the essential rôle of vitamins, amino acids and other dietary factors in nutrition.

The need for standardization of the performance of rats, stressed by Donaldson and his co-workers at the Wistar Institute, has been appreciated by later investigators, but not all studies of growth, body functions and the nutritional value of food constituents have had fundamental significance. Too often, growth data have been of minimum value because diets were deficient in types or proportions of nutrients. It has been pointed out by Wilson (4) and Needham (5) that, because of the quality of the experimental data, the attempts to validate proposed theories of growth have not proved particularly fruitful.

It has been emphasized by Zucker *et al.* (6) that "probably a majority of the formulations of growth for rats have been based upon or fitted to the data of Donaldson, whose weight-time curves were obtained from rats on a diet poorly characterized and certainly deficient in a number of factors." Zucker and Zucker (7) have stated that "most growth data in the literature which have been used by students of growth probably fail to meet good nutritional standards. The whole growth problem has been confused and more or less run into a blank wall by repeated working over of the same old data. There is a fundamental flaw in these data. New data are needed which do meet reasonable nutritional standards, and a useful, if perhaps limited, start can be made with a single species—the rat."

The inadequacy of the earlier standards of growth was appreciated by Mendel and Cannon (8) who stated in 1927 that the improved growth observed by Osborne and Mendel "implies that, owing to the shortcomings of the rations used, the inherent capacity of the rat to grow has in the past rarely been given full

[1] The writers' work has been aided by grants from the Nutrition Foundation, Inc., and the University of California. Early investigations of the growth process were summarized by Brody (1) in 1927. Laboratory studies of the rat prior to 1939 have been reviewed by Friedman (2).

play in the laboratory. Consequently, the published records and compilations of 'norms' fail to furnish an adequate idea of the rate of growth of which the rat is capable."

Although growth of any type or magnitude may serve to determine that under the observed conditions, a given food constituent functions in some capacity essential to the welfare of the organisms, it has become increasingly apparent that it is far more important to determine, if possible, the types and proportions of food elements required for the maintenance of the highest type of physiological performance. This view that optimal performance of animals is the only truly satisfactory standard for use in measuring the biological value of foods appears to be widespread (9–13).

For present purposes, the terms "optimal" and "normal" are considered to be synonymous and in contrast to "average" or "ordinary."[2] The question, "What is normal or optimal growth or what should be the course of growth in the rat strain employed when there are no nutritional deficiencies?" has been raised by Zucker *et al.* (6, 17) who have stated that "If effects of deficiencies are to be elucidated an unhampered norm should first be worked out. We cannot expect growth on deficient diets simply to reproduce normal growth on a smaller scale." Howe (18) has expressed the similar view that "the kind and rate of feeding has such marked influence on the physical development of animals that it is necessary to define normal growth and physique before attempting to discuss the modifying effects of nutrition."

Normal or optimal growth has been defined (14, 15, 16, 19) as that physiological state in which the animal functions in accordance with its design, actual achievements fulfill all the potentialities of the organism and the growth curve is a pure expression of the inherent growth characteristics. Carlson (13) has defined an optimal diet as "that kind and quantity of food which permits and promotes optimum growth, optimum performance of all biologic functions, optimum resistance to disease, optimum conservation of the factors of safety and powers of repair and optimum length of life with optimum efficiency."

Although growth and the physiological sufficiency of diets are measured inadequately by increase in weight (20), or any other single criterion, it appears probable from Thompson's (21) studies of phylogenetic changes and Huxley's (22) investigations of ontogenetic growth that growth is controlled by a recognizable system of forces. The pattern of growth has not been determined accurately but it has been found that growth data obtained in different laboratories for different strains of rats may be correlated conveniently by means of Huxley's equation.[3] The latter has been reinforced and augmented by the investigations of Zucker and co-workers (6, 7, 17, 23).

[2] Some writers (14–16) have inferred that the terms "optical," "normal," "average," and "usual" have equivalent meaning.

[3] A discussion of the older growth equations which have been proposed has been omitted since numerous critical reviews (1, 5, 7, 21, 23, 24–33) are available.

In Zucker and co-workers' useful post-weaning growth equation, $\log W = -k/t + \log A$, k and A are positive constants, t is age in weeks from birth and W is weight in grams. A plot

It has been assumed by some investigators that optimal growth and normal growth may not be synonymous terms. Osborne and Mendel (40) pointed out in 1926 that rapid growth may be indicative of distorted development of organs and body parts and that accelerated growth would be physiologically disadvantageous if time alone were gained at the cost of unorderly, and hence detrimental, development. This problem has been studied for more than three decades in the laboratories at Yale University and the Connecticut Agricultural Experiment Station by Osborne and Mendel and their associates including Anderson, Cannon, Hubbell, Moment, Outhouse, Pickens, Smith and Winters.

The present authors' experiments have been predicated on the assumption that the diet and the rats, described in 1932 by Anderson and Smith (41) as the culmination of the nutrition experiments conducted in Osborne and Mendel's laboratories, were near optimal. It is recognized that, at present, the terms "optimal diet" and "optimal physiological state" have only relative significance and there is no intention to imply that other combinations of dietary components may not be of equivalent, or even superior, quality.

There has been no attempt in this review to record all of the voluminous

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of log W vs 1/t gives a straight line of which k is the slope and, when $1/t = 0$, $\log A = \log W$. The parameters, k and A, permit an analysis of growth in terms of a "growth intensity factor" and an "inherent size factor," respectively. Richards (34) has emphasized Gray's (35) warning that care must be used in interpreting data plotted logarithmically since the diameter of the point on the graph may cover more range than the error of measurement of the variable. It appears that the logarithmic curves of Zucker *et al.* (7) were not subject to this error since it was shown that the relative probable error (ratio of the probable error to the mean) was constant during the whole period of growth.

In Huxley's (22) equation, $\log y = K \log x + \log b$, y is the size or weight of the part, x is the size or weight of the whole, and K (slope of the curve) and b (intercept on the y axis when $x = 1$) are constants. The equivalent expression, $\log y = k_2/k_1 \log x + (\log A_2 - k_2/k_1 \log A_1)$, may be derived from Zucker's equation if the growth of the whole organism is expressed as $\log x = -k_1/t + \log A_1$ and the growth of the part by $\log y = -k_2/t + \log A_2$. The K in Huxley's equation and the ratio k_2/k_1 in Zucker's equation signify that the rate of growth of the part remains constant in respect to the rate of growth of the whole.

Although the constant b is of little biological significance according to Huxley (22) and Needham (36), Zucker *et al.* (7) and Bernstein (33) have pointed out that b is the parameter expressing the ratio of the limiting size of the part to that of the whole, i.e., $b = A_2/A_1^K$. The factors K and b may be useful indices for the characterization and differentiation of species and strains since they are believed to be expressions of the genetic structure and heredity of the organism. Applying Huxley's equation to a large number of vertebrates, Quiring (37) found in 1941 that the K values indicated a fundamental principle of size increase while the b values denoted a scale of being in numerical terms. It might be expected that different strains within the same species would exhibit similar, although smaller, differences.

Zucker *et al.* consider rat growth to be separable into two phases, pre-weaning and post-weaning. For biological reasons, they place the normal post-natal weaning age at 28 days. The heterogonic equation $\log y = k_2/k_1 \log x + (\log C_2 - k_2/k_1 \log C_1)$ was proposed by Huxley (22) and Hamilton and Dewar (38) and it may be derived from Zucker's equation, $\log W = k \log T + \log c$, for the pre-weaning phase of growth or from the related equation of McDowell *et al.* (25). Huxley considered this expression to be in agreement with the work and conclusions of Schmalhausen (39).

literature on the growth of rats on different diets. Consideration has been given, primarily, to the data of the post-weaning period considered to be most highly significant.

TOTAL BODY GROWTH. The data plotted according to Zucker's method in figure 1-A were taken from the papers of Mendel and Hubbell (42) and Anderson and Smith (41). The curves represent the different types of growth obtained in the New Haven laboratories during the period, 1912–1935, with male rats of the same (Yale albino) strain maintained on different diets under essentially the same environmental conditions. The increased rate of growth on the latter diets was accompanied by increased uniformity of growth and by improvement in physiological condition. It was the view of Osborne and Mendel (40) and of Mendel and Hubbell (42) that these effects were due to dietary changes rather than to selective breeding. The superior growth-promoting capacity of the Anderson-Smith diet has been confirmed by Pickens, Anderson and Smith (43). It has been found, also, by Freudenberger (44) that improved diets and care increased the uniformity of growth of rats.

The growth curves obtained by the present authors with the Long-Evans strain of rats maintained on the Anderson-Smith diet are shown in figure 1-B (male rats) and figure 2 (female rats). Other growth curves are given in figure 1 for male rats of the Long-Evans and Wistar strains and in figure 2 for female rats of the Long-Evans strain.

According to Zucker's (45) premises, curves A-3, A-4 and C-2 of figure 1 represent growth on deficient diets. The marked difference between curves C-2 and the two others may indicate, according to Zucker's (17) concept of spontaneous realimentation, that the diets employed differed in respect to the type of nutritional deficiency. The single-straight-line curves A-2, B-2, and C-1 are of the types characterized by Zucker *et al.* (17) as good, normal, optimal and adequate since the growth data obtained with several albino colonies in a number of laboratories yielded straight-line curves of similar slope. Although it was recognized that growth data obtained with diets known to be nutritionally deficient did not yield straight-line curves and that diets which did yield straight-line curves had no pronounced deficiencies, Zucker *et al.* have been careful not to define normal (or optimal) growth as that adhering to their equation. It is of interest that Freudenberger's (46) diet, which yielded straight-line curves (B-2 and C-1 of fig. 1), was later (47) found to be deficient in iodine although the deficiency, as evidenced by rectification, was not sufficient to affect the curve of total body growth.

A type of growth not considered by Zucker and co-workers is represented by curves A-1 and B-1 of figure 1. The difference in slope between the curves A-1 and A-2 for the Yale-albino strain and between the curves B-1 and B-2 for the Long-Evans strain possibly may be explained by the nutritional history of the parent rats. The parents of Anderson and Smith's (41) rats were raised on Mendel and Hubbell's (42) sub-optimal diet which yielded curve A-2 of figure 1. At birth of the young the mothers were placed on the Anderson-Smith diet and at weaning the young were continued on this diet. The young in-

creased rapidly in weight and attained an adult weight optimal for rats of this strain. Similar results were obtained by Pickens, Anderson and Smith (43) under the same conditions. Although their rats at 4 weeks of age were slightly lighter than those of Anderson and Smith, the same adult (16 weeks, post natal) weights were attained by the two groups of rats. On the other hand, curve B-1 represents the growth of rats (maintained on the Anderson-Smith diet) whose

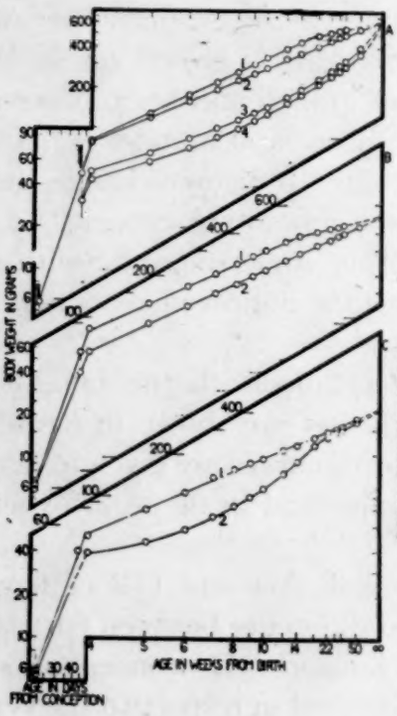


Fig. 1

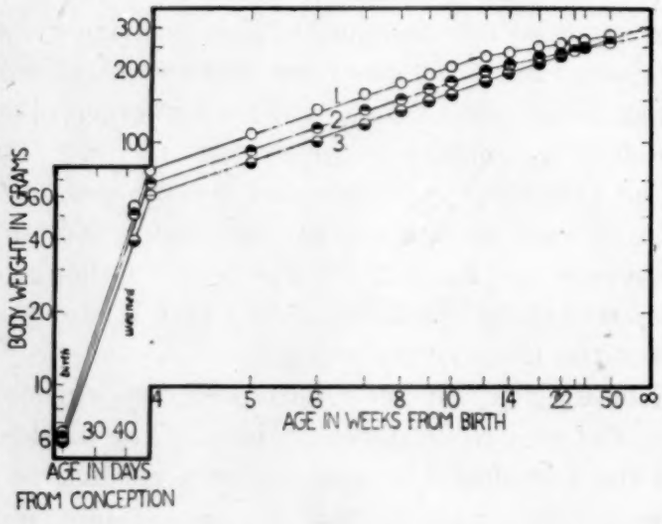


Fig. 2

Fig. 1. Curves showing the relation between body weight and reciprocal age of male rats on various diets. The notations are as follows:

Yale strain. A-1, Anderson and Smith (41), 1932. A-2, Mendel and Hubbell (42), 1935. A-3, Mendel and Hubbell (42), 1925. A-4, Mendel and Hubbell (42), 1919.

Long-Evans strain. B-1, This paper (unpublished data). B-2, Freudenberger (46), 1932.

Wistar strain. C-1, Freudenberger (46), 1932; Greenman and Duhring (60), 1930. C-2, Greenman and Duhring (60), 1923.

Fig. 2. Curves showing the relation between body weight and reciprocal age of female rats on various diets. The notations are as follows:

Long-Evans strain. 1, This paper (unpublished data). 2, Emerson and Evans (56), 1944. 3, Freudenberger (46), 1932.

parents had been raised on the Anderson-Smith diet. If the rats employed by Anderson and Smith had been the progeny of parents which had been raised on the Anderson-Smith diet, it appears probable that a growth curve resembling that found in the authors' laboratory, might have resulted. Hanson and Hayes (48) reported that the birth and weaning weights influenced the adult weight but Anderson and Smith (41) and Zucker *et al.* disagreed with this conclusion. Other workers have found that the adult weight of mice (49), rabbits (50) and Daphnia (51) is not dependent upon the initial growth rate.

If it is assumed that curves A-1 and B-1 of figure 1 represent near-optimal growth it would follow that the single straight-line growth curves are indicative of some dietary deficiency. This hypothesis would seem to be in harmony with the statements of Zucker and Zucker (7) that "the only experimental conditions which can possibly lead to growth according to any relatively simple law require a diet which is adequate for the most rapid growth attainable by nutritional means. Under these conditions only can the growth curve be a pure expression of the inherent growth characteristic of the organism." Since this (or any other) interpretation of the characteristics of optimal growth involve the problem of obesity, the relation of fat deposition to increase in body weight is considered at this point.

It may be inferred that the relatively rapid increase in pre-weaning body weight of rats (curves A-1 and B-1 of fig. 1) raised on the Anderson-Smith or other diet containing a relatively high percentage (20 to 30 per cent) of fat occurs without deposition of fat in excess of the nutritional needs of these animals. Although male rats raised on the Anderson-Smith diet contained a higher percentage of body fat than rats fed the modified (Mendel-Hubbell) Anderson-Smith diet, the data of Pickens *et al.* (43) indicate that the shapes of the body-weight and the fat-free body-weight curves yielded by the two diets were closely similar. The same percentage of body fat is approached as growth on these diets proceeds through maturity. Data in harmony with this observation have been reported by Zucker *et al.* (23) who found that the body weight of rats fed an adequate diet increased slowly up to 70 weeks of age with no evidence (even at 100 weeks of age) of excessive fat deposition, and by Reed *et al.* (52) who observed that the relative distribution of fat in the animal body was the same on high carbohydrate-low fat as on low carbohydrate-high fat diets.[4] The findings of Evans *et al.* (53), Maynard and Rasmussen (54) and Vinson and Cerecedo (55) that lactation is improved on diets containing relatively high proportions of fat may explain the superior quality of such diets for pre-weaning growth.

The straight-line growth curves of the albino colonies considered by Zucker *et al.* were similar in slope. This observation led to the supposition that the slope of the curves provided a mathematical characterization of certain genetical factors, particularly that called "growth intensity." It may be noted from figure 1, however, that the straight-line-portions of different curves may have the same, or different slopes depending, apparently, upon the age and the dietary history of the animals. Zucker *et al.* found less close agreement between the slopes of the growth curves for Long-Evans hybrid rat colonies than for albino rat colonies. These differences were attributed to chance genetical variations resulting from the breeding of hybrid parents but Freudenberger (46) has stated

[4] It has been reported recently by Williams *et al.* (110) that the type of diet [(a) fat, 67.9 per cent of calories; carbohydrate, 9.3 per cent, (b) fat, 38. 6 per cent of calories; carbohydrate, 38.6 per cent, (c) fat, 9.3 per cent of calories; carbohydrate, 67.9 per cent] had little, or no, effect upon the proportion of essential lipids to total lipids. The precentage composition of the essential lipids was nearly the same on diets a and b. The percentage composition of the essential lipids observed on diet c differed from that found on diets a and b.

that even the albinos of the Long-Evans strain resemble the Long-Evans strain as a whole rather than the Wistar albinos from which they were derived. This latter view appears to be supported by the fairly close agreement found for the constants of the equations for body-weight increase (curves shown in fig. 2 and those given by Zucker *et al.*) of the Long-Evans rats investigated by Freudenberger (46), Emerson and Evans (56), Zucker *et al.* (7, 17) and the present authors.

The values 640, 540 and 430 for the males and 380, 300 and 280 for the females found for the limiting weights of the Yale-albino, the Long-Evans and the Wistar strains, respectively, denote fundamental size differences of these strains of rats. In general, the constants, calculated by the present authors from the data reviewed by Zucker and Zucker (7), agree fairly well with the values reported by these investigators. These results would not seem to be in agreement with the observation of Vinson and Cerecedo (55) that "an adult female of the Long-Evans strain weighs less than an adult Wistar female."

The logarithmic-growth curves obtained with rats, both of the Yale-albino and the Long-Evans hooded strains, fed on the Anderson-Smith diet changed slope at about 14 weeks of age (no data were available for the females of the Yale strain). There appears to be some factual basis for the inference that changes in the constants of the growth equation at about 14 weeks of age constitute an objective expression of conditions existing at the beginning of a "normally" occurring adult growth period. It is known that rats become physically mature at about 16 weeks and are usually bred at this age (57) and that volumtary activity increases up to the age of 12 to 18 weeks and then declines (58). An analogous growth equation, which changes slope at birth, applies to the prenatal and the post-natal phases of pre-weaning growth (conclusions based on the data of Stotsenberg (59) and Greenman and Duhring (60)).

A different type of superior growth was observed by Evans (61) with rats which had received injections of hypophyseal extracts. Although the increased growth rate of rats, due to dietary improvement alone, reported by Mendel and Cannon (8) and Anderson and Smith (41) compared favorably with that found by Evans and by Bryan and Gaiser (62) with rats injected with anterior pituitary extract, it appears that the inherent-size differences of the rat strains employed were not taken into consideration. The average of the two log weight-reciprocal time curves given by Zucker and Zucker (7) in representing Evans' data for the 1923–24 series of experiments, is shown in figure 3. Even though the curve may be expressed by Zucker's equation it appears that the pattern of growth has been markedly changed by the injection of pituitary extract. The resulting large animals probably were monstrosities since the weights attained were larger than the near-optimal rats of the same strain and the inherently-larger rats of the Yale-albino strain. Other distortions of the growth pattern resulting from the injection of anterior pituitary growth hormone include relatively greater growth of the viscera and glandular organs (63, 64), and of the liver (63, 65, 66). The occurrence of splanchnomicria in pituitary dwarfism and atrophy of visceral organs after hypophysectomy has also been observed (67).

HETERAUXESIS. It has been generally recognised (18, 22, 68–70) that the tissues and organs of the body develop in definite order and proportion relative to the growth of the body as a whole and that they develop at a rate which, to a considerable extent, is determined by the nutritional state. Many investigators believe that abundant nutrition promotes the maximum expression of inherited characteristics while under-nutrition results in retarded development. It seems apparent, however, that many studies on heterogonic growth are subject to the criticism that sub-optimal diets, utilized purposefully in some cases, were employed. Such data may be of value, however, since they may serve to characterize sub-optimal growth.

Skeletal growth, growth of some of the principal organs and changes in body composition with age are discussed in the following sections.

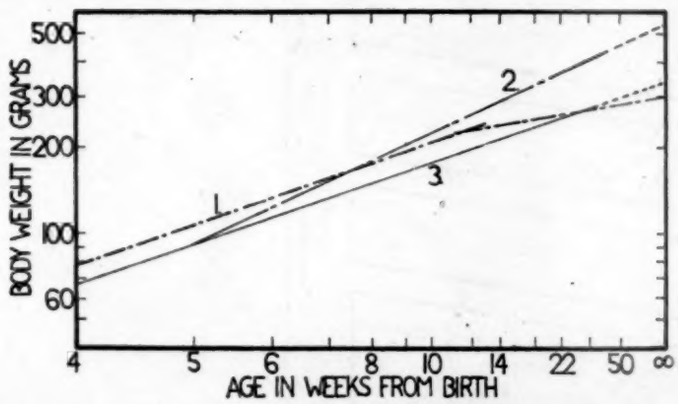


Fig. 3. Curves showing the relation between body weight and reciprocal age of female rats injected with hypophyseal extract. The notations are as follows:

Long-Evans strain. 1, This paper (unpublished data, Anderson-Smith diet). 2, Evans (61), 1923–24. Rats injected with hypophyseal extract. 3, Evans (61), 1923–24. Control rats.

1. *Skeletal growth.* The skeletal growth of male rats of the Yale strain, raised on the diet employed by Osborne and Mendel (40) in 1926, was investigated by Outhouse and Mendel (71) in 1932–33. "Rapid" growth of about 4 grams per day and slow growth of about 2 grams per day were maintained by varying the supplements of yeast and lettuce. Although these investigators found that the general body proportions were nearly the same for both groups of rats, they concluded that body length and weight were more closely correlated than body length and age. At a given body weight the leg bones of the slower-growing (older) rats were longer and chemically more mature than those of the faster-growing (younger) animals. These differences diminished with increasing age and the leg bones of 420 gram rats, both fast- and slow-growing, had the same linear dimensions.

Outhouse and Mendel, as well as Moment (72) who found a similar growth response on the same diets, believed that the growth of their rats was normal on both diets. It would appear, however, from the log-reciprocal plots of Outhouse and Mendel's data (fig. 4) that both diets were sub-optimal, although the diet fed to the faster-growing group (fig. 4-A-1) was less deficient than that supplied to the

slower-growing group (fig. 4-A-2). It may be noted from the log-reciprocal curves shown in figure 4-C and 4-D that the body-length (axial skeleton) and femur-length (appendicular skeleton) curves approach more closely a straight line for rats of the Long-Evans strain fed on the Anderson-Smith diet than for rats of the Long-Evans and the Yale strains fed on other diets.

The influence of age upon the growth of the leg bones is shown graphically by the curves (fig. 5) relating body length and femur length in millimeters. The femurs of Outhouse and Mendel's rats were longer for the slower-growing (older) than for

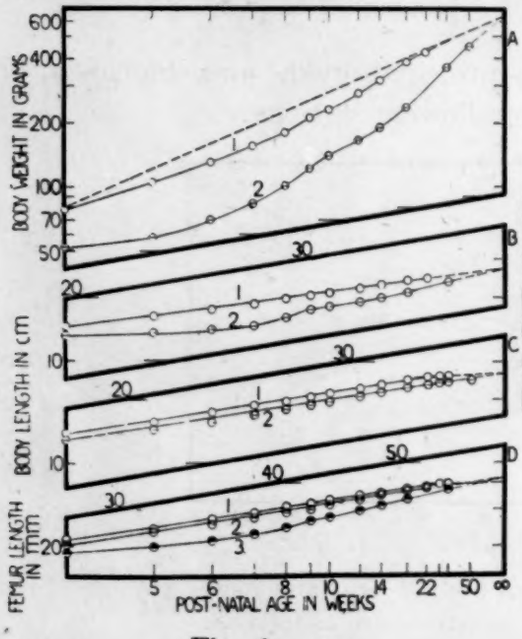


Fig. 4

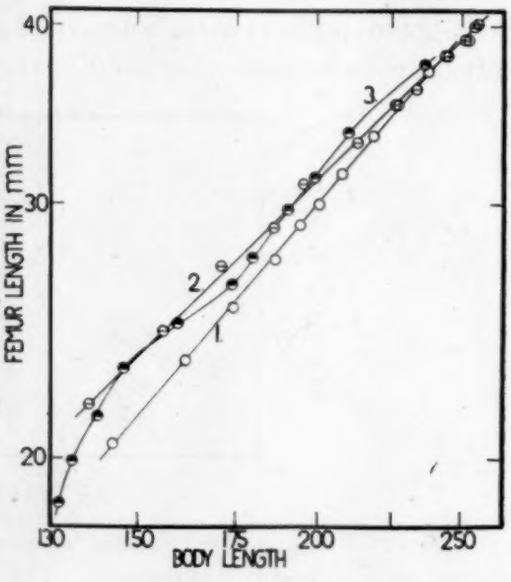


Fig. 5

Fig. 4. Curves showing the relation between reciprocal age and body weight, body length and femur length of male rats on various diets. The notations are as follows:

Yale strain. A-1, B-1 and D-2, Outhouse and Mendel (71), 1932–33. Rapid group. A-2, B-2, and D-3, Outhouse and Mendel (71), 1932–33. Slow group.

Long-Evans strain. C-1 and D-1, This paper. C-2, Freudenberger (46), 1932.

Fig. 5. Curves showing the relation between the femur length and the body length of male rats on various diets. The notations are as follows:

Yale strain. 1, Outhouse and Mendel (71), 1932–33. Rapid group. 3, Outhouse and Mendel (71), 1932–33. Slow group.

Long-Evans strain. 2, This paper.

the faster-growing (younger) group of the same body length. Similar (tachyauxetic) growth curves of the femurs of Wistar-albino rats result from plots of the data obtained by Hammett (73) on Greenman and Duhring's (60) deficient diets. The tachyauxesis of the femur growth observed for rats maintained on deficient diets is in harmony with the earlier report of Winters *et al.* (69) that "while the (deficient) animals made only 8.7 to 19.5 per cent of the normally expected gain in body length, the leg bones made 36 to 41 per cent." No analogous data are available for rats of the Yale and the Wistar strains raised on the Anderson-Smith diet but the authors' data for rats of the Long-Evans strain are shown in figure 5-2. It may be noted that growth of the femur relative to that of the axial skeleton was both uniform and isauxetic (K = 1).

It may be concluded from the lateral diameters, the anterior-posterior diameters and the epiphyseal widths of the femurs found by Outhouse and Mendel for Yale rats and by the authors (unpublished) for Long-Evans rats that the thickness of the femurs at a given femur length was greater for the higher- than for the lower-quality diets. Many investigators have reported that the skeleton grew persistently (43, 68–70), but variably (69), on deficient diets. That the curves (fig. 4) of body length and femur length approach more closely to a straight line than those of body weight, is considered to be evidence in harmony with this observation.

It may be concluded that the skeletons of rats raised on the Anderson-Smith diet grow without apparent abnormalities.

2. *Organ growth.* In Moment's (72) investigation of the growth of the principal organs and glands, the strain of rats (Yale) was the same as that employed by Outhouse and Mendel in studying skeletal growth. The diets were similar to those utilized by Outhouse and Mendel. Moment concluded that the size of three different muscles, the kidney, the spleen, the thyroid and the pituitary was more closely correlated with body size than with age. The same correlation was observed for the heart and liver although there was a tendency, at the same body weight, for the rapid-growing group to have larger hearts and livers than the slow-growing group. The growth of the eyeball seemed to be correlated with age rather than body size. At a given body weight, the size of the thymus was greater for the rapid-growing than the the slow-growing rats although the age at which maximum size was attained and began to diminish was the same for both groups.

The size of various organs and glands of the Long-Evans and the Wistar rats at birth, and at 3, 12 and 52 weeks of age was investigated by Freudenberger (46) while data for most of the organs and glands of Wistar rats, based largely on the work of Hatai (74) and Jackson (75), were tabulated by Donaldson (3).

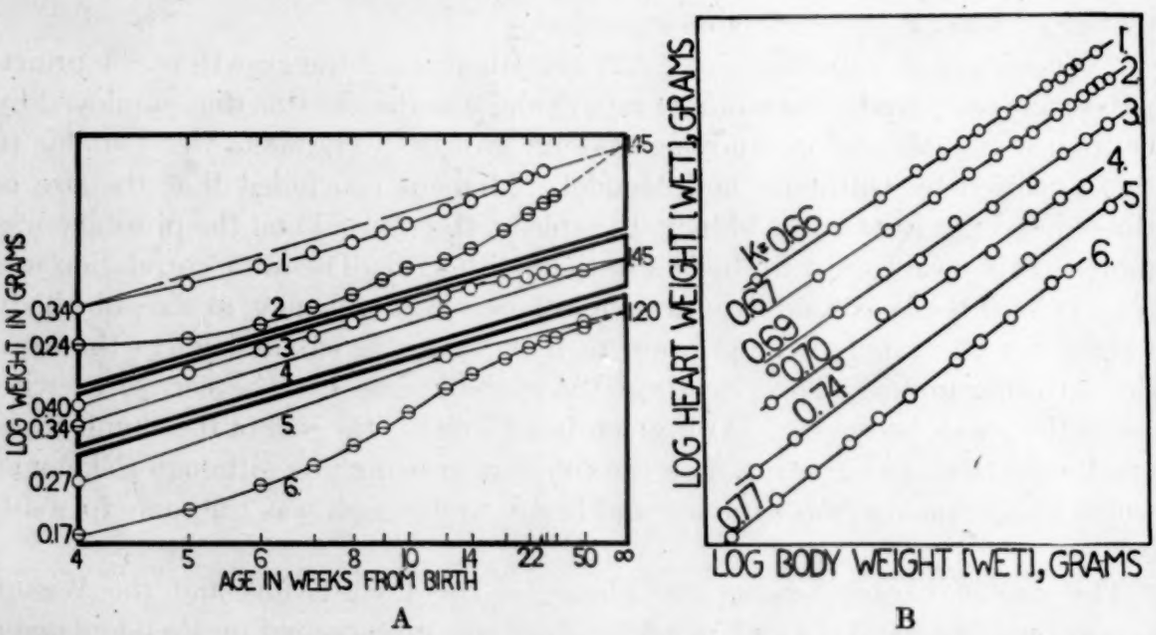
(a) *Heart.* It may be observed from the log heart weight-reciprocal age curves shown in figure 6-A that, according to Zucker's premises, the growth of the heart was sub-optimal for the Yale and the Wistar strains of rats fed on the (presumably inadequate) diets of Moment and Donaldson. It is of interest that growth of the heart appeared to be near-optimal for the Wistar and the Long-Evans strains of rats maintained on the (presumably) adequate or near-optimal diets of Freudenberger and the authors. Even though a reasonably large number of animals was employed, the data reported by Freudenberger for Wistar rats sacrificed for organ measurements and the data reported for his Wistar colony rats were not entirely consistent. For this reason, it is considered that the change in slope of the curve for Freudenberger's Wistar rats may be an artifact.

Log heart weight–log body weight curves are shown in figure 6-B. The points for each strain of rat and for both types (adequate and inadequate) of diets fall reasonably close to a straight line. The lack of any significant difference between the two curves of each pair of curves for the Long-Evans, the Yale and Wistar strains may indicate that, relative to total body growth, the growth of the heart (a vital organ not directly concerned with the metabolism of food) was not measurably influenced by the different diets employed by the investigators

whose experimental data have been recorded. There is some indication, however, of a strain difference in the growth of the heart relative to increase in body weight since the average slopes (K) of the curves were 0.66, 0.70 and 0.76 for the Long-Evans, the Yale and the Wistar strains, respectively.

It appears that Moment's view that, for a given body weight, the heart tended to be slightly larger for a rat which grew rapidly than for one which grew less rapidly, is not substantiated by the data discussed in this section.

It may be concluded that the heart of a rat raised on the Anderson-Smith diet grows without apparent abnormality.



A B

Fig. 6-A. Curves showing the relation between the heart weight and reciprocal age of male rats on various diets. The notations are as follows:

Yale strain. 1, Moment (72), 1933. Rapid group. 2, Moment (72), 1933. Slow group.

Long-Evans strain. 3, This paper. 4, Freudenberger (46), 1932.

Wistar strain. 5, Freudenberger (46), 1932. 6, Donaldson (3), 1924.

Fig. 6-B. Curves showing the relation between the heart weight and the body weight of male rats on various diets. The notations are as follows:

Long-Evans strain. 1, Freudenberger (46), 1932. 2, This paper.

Yale strain. 3, Moment (72), 1933. Rapid group. 4, Moment (72), 1933. Slow group.

Wistar strain. 5, Freudenberger (46), 1932. 6, Donaldson (3), 1924.

(b) *Kidney and liver.* The log kidney weight-reciprocal age curves shown in figure 7-A resemble closely the comparable curves representing heart growth given in figure 6-A. It appears that growth of the kidney was sub-optimal on the inadequate diets and that growth of the kidney on the adequate or near-optimum diets conforms satisfactorily to Zucker's long-weight-reciprocal time equation. It is evident, also, from an inspection of the log kidney weight-log body weight curves (not shown) constructed from these data that kidney growth was more uniform on the higher-than on the lower-quality diets.

It may be observed that the similar liver-growth curves (curves 3, 4 and 5 of fig. 7-B) derived from adequate and near-optimal diets change abruptly in slope

at about 12 weeks of age. This change is considered to be real and not artifactitious since it has been observed not only for liver weight (fig. 7-B) but also for kidney weight (fig. 7-A), body length (fig. 4-C-1), femur length (fig. 4-D-1), and total body weight of female (fig. 2-1 and fig. 2-2) and male (fig. 1-A-1, and fig. 1-B-1) rats.

It is of further interest that the total body and the organs (heart, kidney, and liver) considered as well as the femur and the body have limiting weights or lengths which are characteristic of each of the three strains of rats investigated. These data are given in table 1. It should be emphasized that each figure in the

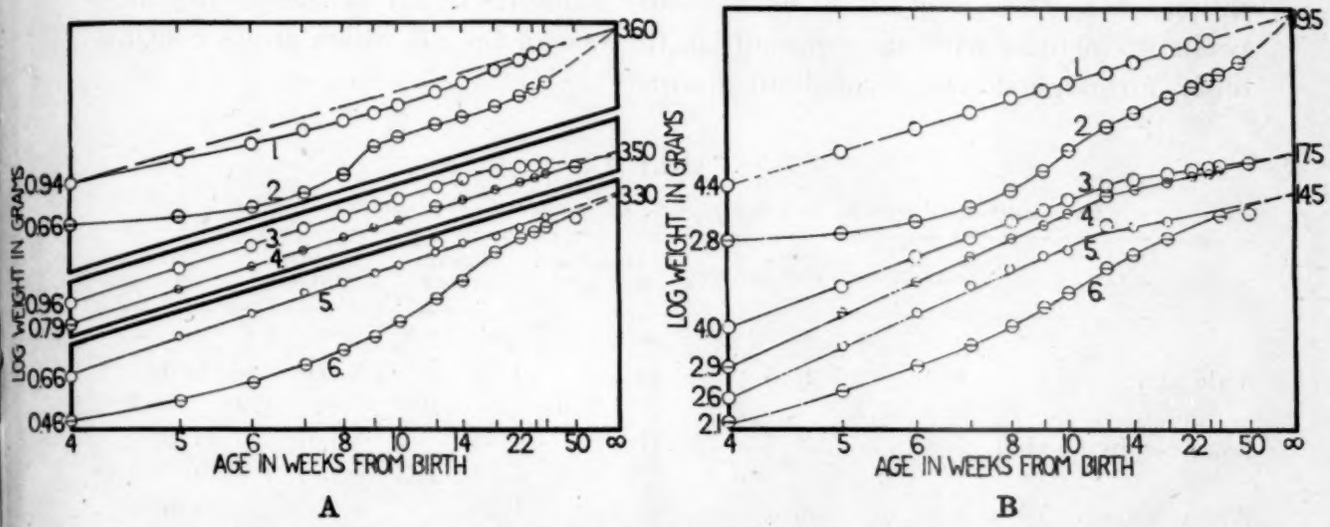


Fig. 7-A. Curves showing the relation between kidney weight and reciprocal age of male rats on various diets. The notations are as follows:

Yale strain. **1, Moment (72), 1933. Rapid group. 2, Moment (72), 1933. Slow group.**
Long-Evans strain. **3, This paper. 4, Freudenberger (46), 1932.**
Wistar strain. **5, Freudenberger (46), 1932. 6, Donaldson (3), 1924.**

Fig. 7-B. Curves showing the relation between liver weight and reciprocal age of male rats on various diets. The notations are as follows:

Yale strain. **1, Moment (72), 1933. Rapid group. 2, Moment (72), 1933. Slow group.**
Long-Evans strain. **3, This paper. 4, Freudenberger (46), 1932.**
Wistar strain. **5, Freudenberger (46), 1932. 6, Donaldson (3), 1924.**

table was derived from two independent groups of rats of the same strain maintained on different diets in the same or different laboratories.

3. *Body composition.* There has been considerable interest during the past two decades in the growth (increase) of chemical constituents of the animal body with increasing age. Needham (5, 36) has reviewed and correlated a large amount of data on many types of animals and he has proposed what has been called the "chemical ground plan of animal growth." While Needham was concerned, primarily, with embryonic growth, the present discussion deals only with the post-weaning phase of the growth of the rat. Chanutin (76) concluded in 1931 that "chemical maturity" of the rat is reached approximately at the age of weaning (28–30 days) although Moulton (77) had estimated, nearly ten years earlier, that rats become chemically mature at about 50 days of age. The major-

ity of the literature data have been on the chemical groups, protein, fat and ash, although water, chemical elements, creatine, glycogen and other entities have been investigated. The authors' present interest is limited to protein, fat, ash and water.

Earlier investigations (43, 78–81) of the change in chemical composition with age have led to the view that the gain in weight of young growing animals results in large part from accretions of protein, water and ash. It has been observed, as age and live weight increase, that the proportions of protein and water in the animal tend to decrease and the proportion of fat to increase. The proportion of minerals may decrease slightly but does not change greatly under usual conditions. On the other hand, the absolute amounts of all of these substances probably increase with age especially in the case of the rat which grows continuously, although slowly, throughout maturity.

TABLE 1

Limiting size of body and body parts of male rats of different strains

STRAIN	BODY WEIGHT		BODY LENGTH		FEMUR LENGTH*		HEART WEIGHT		KIDNEY WEIGHT		LIVER WEIGHT	
	gm.	*S.R.*	*gm.*	*S.R.*	*gm.*	*S.R.*	*gm.*	*S.R.*	*gm.*	*S.R.*	*gm.*	*S.R.*
Yale albino	640		27.5		44		1.45		3.60		19.5	
		7.5		3.7		5.0		0		1.2		3.6
Long Evans hooded	540		26.5		41		1.45		3.50		17.5	
		6.2		6.3		3.3		7.3		4.3		5.6
Wistar albino	430		23.5		39		1.20		3.30		14.5	

*Air-dried. All other values wet weight.

S.R. is the significance ratio (difference divided by the probable error of the difference). If the quotient is greater than 3 the difference is considered significant.

The effect of the plane of nutrition on body composition is illustrated by McMeeken and Hammond's investigations of swine, reviewed by Howe (18), and by Moulton, Trowbridge and Haigh's (80) studies of cattle in which different nutritional levels were maintained by controlling the quantity of food ingested. Similar data for the body composition of rats have been reported by Pickens, Anderson and Smith (43) who maintained different nutritional levels by controlling the quality of diets fed *ad libitum*. Essentially the same relations of protein, fat, ash and water were found for both species of animals and for both types of nutritional control.

Moulton (77) expressed the opinion that the plane of nutrition would not affect the composition of animals calculated on a fat-free basis unless the conditions were such as to cause retardation of development. A similar view was advanced by Pickens, Anderson and Smith who found that the whole bodies varied considerably in composition at 230 days of age but that the fat-free material of all groups was practically of identical composition. Different degrees of correspondence were observed, however, over the experimental period from weaning to 230 days. It has been found from semi-logarithmic plots of the

TABLE 2

Percentage of body components of male rats on various diets

WATER			PROTEIN			ASH			FAT			BODY PORTION	DIET	RAT STRAIN	REFERENCE
Days†			Days			Days			Days						
37	90	127	37	90	127	37	90	127	37	90	127				
73	70		21	24		4.4	5.1		1.7	0.9		Vital	‡	Wistar	Nash (82)
67	66	65	15	15	16*	3.0	3.8	3.8	12	12	13	Body	Gliadin	Yale	Pickens *et al.* (43)
69	67	66	16	18	18	2.9	3.8	3.8	7.9	8.0	9.6	Body	Lactalbumin	Yale	Pickens *et al.* (43)
67	63	61	16	18	19	2.4	3.0	3.2	12	13	14	Body	Stock	Yale	Pickens *et al.* (43)
65	60	57	16	16	16	2.2	2.5	2.6	14	19	22	Body	Anderson-Smith	Yale	Pickens *et al.* (43)
		70			21			2.7			4.9	Body	Low-salt	Yale	Light *et al.* (82)
		64			20			3.9			11	Body	Calorie control	Yale	Light *et al.* (82)
68		62	17		19	2.4		3.4	12		15	Body	Normal control	Yale	Light *et al.* (82)
60	63		15	17		1.5	1.3		20	18		Reducible	‡	Wistar	Nash (83)

* Changed to lactalbumin diet at 110 days of age.

† Postnatal age.

‡ Described as "supermaintenance diet," composition not given.

TABLE 3

Percentage (fat-free) of body components of male rats on various diets

WATER			PROTEIN			ASH			BODY PORTION	DIET	RAT STRAIN	REFERENCE
Days†			Days			Days						
37	90	127	37	90	127	37	90	127				
75	71	70	21	24	24	4.5	5.2	5.2	Vital	‡	Wistar	Nash (83)
76	75	75	17	17	18*	3.4	4.3	4.4	Body	Gliadin	Yale	Pickens *et al.* (43)
75	73	73	18	19	20	3.2	4.1	4.2	Body	Lactalbumin	Yale	Pickens *et al.* (43)
76	72	71	18	21	22	2.7	3.5	3.7	Body	Stock	Yale	Pickens *et al.* (43)
76	73	73	19	20	20	2.6	3.1	3.3	Body	Anderson-Smith	Yale	Pickens *et al.* (43)
		74			22			2.8	Body	Low salt	Yale	Light *et al.* (82)
		72			22			4.4	Body	Calorie control	Yale	Light *et al.* (82)
77		73	19		22	2.7		4.0	Body	Normal control	Yale	Light *et al.* (82)
75	77		19	21		1.9	1.6		Reducible	‡	Wistar	Nash (83)

* Changed to lactalbumin at 110 days of age.

† Postnatal age.

‡ Described as "supermaintenance diet," composition not given.

data of Pickens *et al.* that the relation between the various components on the different diets was the same whether calculated on a total-weight or a fat-free basis. Differences in composition were of reduced magnitude when calculated on the latter basis. Examination of the plots reveals that the rats fed the Anderson-Smith diet had higher fat content of their bodies and more nearly optimal skeletal growth (ash) than rats maintained on deficient diets.

It was concluded by Light, Smith, Smith and Anderson (82) in 1934 that, under unfavorable nutritive conditions, the chemical composition of the body may be varied so that the available food material is best used to support the vital equilibria and to maintain the essential tissues. This view that changes in body composition may reflect an "adaptation to deficient diets" was reiterated in the later studies of Pickens, Anderson and Smith (43). The general concept that there may be a "vital portion" and a "reducible portion" of the animal body has been discussed more recently by Nash (83). This hypothesis is somewhat analogous to that proposed in 1919 by Terroine (84). The vital portion was considered to be the part necessary for life and the reducible portion the part normally in excess of the minimum requirement for existence. There was assumed to be a vital and reducible portion of each chemical constituent equivalent, respectively, to the amount present at death from inanition and the amount lost during the period of inanition leading to death. Nash reported data illustrating the characteristics of the vital and reducible portions of Wistar rats and he concluded that these body portions differed in composition. On the basis of this concept, it may be assumed that the body composition of rats on deficient diets would resemble more closely the composition of the vital than the reducible portion.[5]

Values for the percentage composition of water, protein, ash and fat in the vital and reducible portions of rats calculated from Nash's data and in the bodies of rats calculated from the data of Pickens *et al.* (43) and Light *et al.* (82) are shown in tables 2 and 3. Although both the Yale and Wistar strains of rats were employed in these studies, definite strain differences in respect to chemical composition have not been demonstrated unequivocally even though the possibility that such differences may be found is indicated by the work of other investigators. Strain differences of rats in response to dietary choline (85), biotin (86,87), lactose (88,89), glucose (90–94) and the B-vitamins, minerals and proteins (95) have been reported.

[5] It may be that the method employed by Nash does not give a true picture of changes in ash content of animals maintained on inadequate diets. The decrease in skeletal material in starvation may not be equivalent to the increase on a poor diet although the ash values may approach each other. Nash has stated that "it is conceivable that by varying the inanition diet, e.g., by restricting in the diet only the constituent considered, the reducible portion of the constituent may be found to be larger and the vital portion smaller than these respective portions as determined by the effects of a complete inanition diet, since on a complete inanition diet death may occur due to the insufficiency of an essential chemical other than the particular constituent under consideration." He pointed out, in addition, that death during starvation is not necessarily due to loss of body substance *per se*, but may be caused by inability to maintain physiological equilibria.

TABLE 4

K values (slopes) of heterauxetic curves for body components of male albino rats on various diets

TOTAL-WEIGHT BASIS				FAT-FREE BASIS			BODY PORTION	DIET	RAT STRAIN	REFERENCE
Water	Protein	Ash	Fat	Water	Protein	Ash				
0.97	1.15	1.13	0.63	0.97	1.15	1.11	Vital	*	Wistar	Nash (83)
0.98							Body	Not given	Wistar	Lowrey (105)
0.97							Body	Not given	Not given	Hamilton and Dewar (38)
0.97		1.11	0.75	0.93		1.09	Body	Not given	Wistar	Hatai (109)
0.97	1.15	1.28	1.09	0.97	1.15	1.28	Body	Gliadin	Yale	Pickens *et al.* (43)
		0.88				0.93				
0.93	1.23	1.33	1.43	0.97	1.15	1.40	Body	Calorie control	Yale	Pickens *et al.* (43)
	0.98	0.82				0.90				
0.95	1.11	1.07	1.10	0.97	1.13	1.07	Body	Control	Wistar	Nash (83)
	1.11	1.00					Body	Not given	Not given	Buchner and Peter (106)
		1.43								
0.95	1.11	1.04	1.54	0.97	1.13	0.93	Body	Not given	Not given	Chanutin (76)
						1.26				
0.95	1.11	1.28	1.11	0.95	1.13	1.33	Body	Stock	Yale	Pickens *et al.* (43)
		0.74	1.84			0.87				
0.92	1.07	1.19	1.17	0.95	1.11	1.35	Body	Anderson-Smith	Yale	Pickens *et al.* (43)
			1.80							

* Described as "supermaintenance diet," composition not given.

It may be concluded from the data in table 2 that, as the efficiency of the diet increases, the composition of the body becomes less similar to that of the vital portion and more similar to that of the reducible portion. On a fat-free basis (table 3), the percentage of water and protein is essentially the same for all diets but that of ash is inversely related to the efficiency of the diet. Additional information is yielded by consideration of the curves of growth and heterogony derived from Huxley's heterauxetic equation which has been employed by Needham and other investigators (5, 23, 36, 38, 83, 96). Some of the data examined indicate that on adequate diets the increase of chemical constituents with time follows this equation at least up to maturity. The data are not extensive, however, and this problem has not been studied intensively although there is some indication that "disturbances" occur particularly during transition periods between growth phases (pre-weaning and post-weaning growth periods). The curves obtained by Nash who plotted the growth of the elements of the vital portion against the vital portion as a whole, are of particular interest since nutritionally limited growth should resemble that of the vital portion.

In table 4 are listed values for the slopes of the straight-line curves obtained from plots of the logarithm-substance weight against the logarithm-body weight both on the wet-weight and the fat-free basis. These values are given in the order of decreasing similarity to the slopes calculated by Nash for the water and protein components of the vital portion. In some cases a single straight line is inadequate to represent all of the data. Needham (5) pointed out that heterauxetic curves which are not single straight lines may occur but he excluded curves of this type from his discussion. It has been found in the present review that many of the variant curves may be attributed to nutritional disturbances. The K value for each section of such curves is shown in the table.

It is of interest that of the diets employed by Pickens, Anderson and Smith the Anderson-Smith diet was the only one which yielded a single straight-line heterauxetic curve for body ash and it may be inferred from the data given in table 4 that the proportion of water, protein and ash to total body weight increased with an efficiency which was at least as great on the Anderson-Smith as on the other diets. It may be concluded that rats raised on the Anderson-Smith diet are not abnormal in the proportions of water, protein and ash to total body weight on a fat-free basis.

PHYSIOLOGICAL STATE. It has been pointed out, previously, in this paper that the ideal conception of optimal growth would include optimal reproductive ability, optimal resistance to disease and optimal longevity. Although McCay (97) has found that increased rate of growth may be accompanied by decrease in longevity, his observed longevity was not related to optimal resistance to unfavorable environmental conditions. No reports are available on the longevity or the resistence to disease of rats raised on the Anderson-Smith diet.

In table 5 are presented data on the reproductive performance of the Yale, the Long-Evans and the Wistar strains of rats maintained on various diets. It appears evident from these figures that the reproductive performance was best on the Anderson-Smith diet. In view of the findings of Griffith and Farris

(57), Greenman and Duhring (60), Morrison (98), Fitzhugh *et al.* (99), Templeton *et al.* (100) and Emerson and Evans (56), it may be found that reproduction beyond first litters may not be as satisfactory because of the relatively high fat (lard) content of the Anderson-Smith diet. Experiments designed to test this hypothesis are in progress in the authors' laboratory.

Exercise is included among the more important environmental factors which, in addition to diet, may influence growth and physiological well-being of experimental animals. Since all of the rats considered in this review were housed in stationary cages, there is no information available on the extent to which the growth and reproduction data obtained with the Anderson-Smith and other

TABLE 5

Average reproductive performance of different strains of rats on various diets

FEMALES FERTILE	YOUNG					STRAIN	REFERENCE
	Per litter	Birth weight	Weaned†	Weaning weight			
				Males	Females		
per cent	*number*	*gm.*	*per cent*	*gm.*	*gm.*		
86	7.2		71	23	26	Yale	Mendel and Hubbell (42), 1912
65	6.3		67	31	31	Yale	Mendel and Hubbell (42), 1919
68	6.4		76	31	30	Yale	Mendel and Hubbell (42), 1925
93	9.6	5.8	90	48	47	Yale	Mendel and Hubbell (42), 1935
81	5.9	5.8	82	43‡		Long-Evans	Evans and Bishop* (107), 1923
	6.7	5.9		40	39	Long-Evans	Freudenberger (46), 1932
95	9.3	6.3	100	56	54	Long-Evans	This paper*
	6.1	4.2		20	22	Wistar	Donaldson* (3), 1924
81	8.2	4.8	67	29‡		Wistar	Greenwood* (108), 1940
	8.5	5.4		41	38	Wistar	Freudenberger (46), 1932

* First litter data. Litter number not specified in other cases.
† Refers to the 6 or 7 young retained of the total litter.
‡ Average of young weaned. Figures not given for each sex.

diets would have been altered by voluntary exercise. The conclusions on this point from published data are conflicting. It was reported by Hatai (101) that the heart, liver and kidneys of exercised rats were heavier than the same organs of non-exercised animals though there was little, if any, difference in body length and body weight. The spleen and the lungs of the non-exercised rats were found to be heavier than these organs of the exercised animals. Hatai's observation that exercise prevented, or at least delayed, the onset of a prevalent pulmonary infection in the albino rat may be interpreted to mean that the heavier lungs of the unexercised rats resulted from infection rather than from any normal physiological variation. Seven generations of rats were studied by Donaldson (102) who found that exercised males were slightly lighter and exercised females somewhat heavier than the non-exercised animals although

the effect was not cumulative from generation to generation. Body length was not appreciably affected, but the heart, kidney, suprarenals and gonads of the exercised rats were somewhat larger than these organs of the non-exercised animals. Donaldson attributed the decrease in body weight of exercised males to loss of body fat and this conclusion was corroborated by Reed, Yamaguchi, Anderson and Mendel (52). Borovansky (103) reported that the body weight both of male and female rats was heavier for the exercised than the non-exercised animals. Cages facilitating voluntary exercise were recommended by Greenman and Duhring (60) who stated that, "as a result of a highly varied diet, gentling and exercise, it is not only possible to produce heavier animals but their fertility also may be greatly increased." Some evidence indicating decreased longevity of exercised rats has been presented by Slonaker (58).

The effect of food intake, activity and temperature upon the body weight of twelve female albino rats of the Sprague-Dawley strain has been studied recently by Brobeck (104). It was concluded that (a) there was a negative correlation between weight change and activity when food intake and environmental temperature were constant, (b) increasing the food intake increased body weight gain when activity and environmental temperature were constant, and (c) weight loss was less at 86° than at 70°F. when food intake and activity were constant. The following criticisms of these carefully controlled experiments might be offered. Weight gains would not be comparable at different ages (4 to 10 months) of rats since growth on the experimental diet, even of the non-exercised rats, was sub-optimal. It would be of interest, and possibly of increased physiological significance, to carry out this same experiment with a near-optimal ration fed *ad libitum*.

SUMMARY

Rats, rat organs and chemical components of rats grow (increase in weight or other dimensions) at rates or in proportions which are considered to be sub-optimal, adequate or near-optimal. The straight-line curves derived from plots of the logarithms of weights or lengths and the reciprocals of time according to the equations of Zucker *et al.* appear to provide satisfactory, although empirical, bases for the evaluation of growth of rats and the relative efficiency of experimental diets. Rats of the Yale, the Long-Evans and the Wistar strains appear to have widely different limiting weights which represent strain differences necessary to consider in growth experiments. Although voluntary exercise and temperature affect gain in body weight of female rats significantly on sub-optimal diets it has not been determined to what extent growth on near-optimal diets would be influenced by these factors. No abnormalities in the growth of rats, rat organs and chemical components of rats maintained on the Anderson-Smith diet have been observed. It appears that the Anderson-Smith diet is superior to the other diets for which experimental data are available in promoting high quality growth and reproductive performance of rats.

It has been concluded as a working hypothesis that the Anderson-Smith diet contains the types and proportions of chemical substances which are near-

optimal for the growth of body, organs and body components and for the reproduction of the rat. No information is available on the efficacy of this diet in promoting longevity, resistance to disease and physiological well-being as determined by other criteria.

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DEVELOPMENT OF THE GRAAFIAN FOLLICLE AND OVULATION

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Twenty years have passed since it was discovered that ovulation could be induced in sexually immature rats and mice by making single or repeated implants of pituitary tissue (Smith, 1926; Aschheim, 1926; Zondek, 1926). Also, a similar period has elapsed since a practical laboratory technique was devised for hypophysectomizing rats (Smith, 1927). These two events gave impetus to an enormous amount of research on pituitary-ovarian relationships. Many species of vertebrate animals, ranging from fishes to mammals, have been used in experiments on ovulation and the gonadotropic preparations employed have been equally diverse. Much of this work has been of an empirical nature and further complicated by wide variation both in methods used for preparing and standardizing gonadotropic materials and experimental procedures. This makes it difficult, if not impossible, to find a common ground for the comparison of results.

The object of this review is to discuss the normal processes involved in the development and ovulation of a Graafian follicle with particular emphasis on the parts played by the pituitary and ovarian hormones. For this purpose, a rather arbitrary selection from the extensive literature dealing with this subject was made and only those papers were included that have a direct bearing on the development of the topic. The author is aware that this may lead to errors of omission and consequently, wherever possible, works of general nature are included from which additional references may be obtained.

FOLLICULAR DEVELOPMENT. Ovulation, in mammals, marks the end of the follicular phase of the estrous cycle and the beginning of the luteal phase. When considered from the standpoint of probable evolutionary significance the luteal phase is a specialization. Ovulation is not followed necessarily by the development of a corpus luteum and when such occurs it is not always associated with viviparity. Corpora lutea are not present in amphibia but are found in many species of oviparous, ovoviviparous and viviparous elasmobranch fishes and reptiles. Such corpora are not essential for the maintenance of pregnancy nor is it known that they secrete progesterone. Therefore, neither luteinization nor the presence of progesterone, at least in vertebrates other than mammals, is an indispensable requirement for ovulation.

On the other hand, the factors that control development of the follicle and ovulation seem to be fundamentally the same, if not identical, in all vertebrates. The physiological processes involved in the follicular phase of the estrous cycle of mammals are apparently homologous with those responsible for growth of follicles and ovulation in species of vertebrates in which a luteal phase is lacking. Thus, the available evidence indicates that in this regard all vertebrate animals are basically alike.

Ovarian function in the adult animal is under the direct hormonal control of the anterior lobe of the pituitary gland (Smith, 1939) and the two active agents involved are the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) (Fevold, 1939, 1943; Chow, 1943). The ability of the ovaries to respond to these pituitary gonadotropins is attained as the animal aproaches sexual maturity while the ovaries of the very young animal are refractory. It is also a common observation that even in the adult animal all the follicles are not equally responsive. When a pituitary extract is given in small doses to a rat the effects may be limited almost entirely to the larger follicles, but if the dosage is increased, smaller follicles are called into the reaction, while the primordial follicles fail to be stimulated even when large amounts of the preparation are injected. This raises the question as to the point at which, in the life of a follicle, it becomes responsive to gonadotropic hormones and thus comes under pituitary control. Therefore, some consideration should be given to the conditions governing follicular growth since they determine whether or not development is terminated by a successful ovulation.

One of the striking features of the adult ovary is the presence of tissues that have retained their embryonic characteristics. The cells of the germinal epithelium, in probably all vertebrates, are capable of active mitoses throughout the reproductive life of the animal. This tissue seems to be homologous with the coelomic epithelium and either lies entirely on the surface of the ovary or, as is more usual, is represented by tissue in the ovarian cortex which was derived earlier from the surface epithelium (Mossman, 1938). The embryonic nature of the germinal epithelium is also shown by the fact that oogenesis may continue even after sexual maturity (Hartman, 1939; Mossman, 1937).

The primordial follicles, whether they are budded off directly from the germinal epithelium on the surface of the ovary or are formed in the underlying ovarian cortex, at first do not have a theca interna. Such follicles consist of an oocyte surrounded by a single layer of granulosa cells. When the oocyte begins to grow, a theca interna is formed from the contiguous connective tissue. As the theca thickens by hyperplasia and the addition of more cells from the stroma, it is provided with vascular loops which cover the follicle but do not enter the granulosa. Correlated with this is an increase in mitotic activity in the granulosa and the ovum becomes situated in the center of a solid ball of cells several layers in thickness.

Oogenesis and follicular development in mammals can proceed up to this point in the absence of pituitary hormones. In the immature hypophysectomized rat follicles may develop to the stage of beginning antrum formation (Smith, 1930) and the total number of primordial follicles may be greater than normal (Swezy, 1933). Hypophysectomy of the adult animal leads to rapid atresia of all of the large antra-containing follicles, while the small follicles without antra may show no effects. This seems to be the general situation among mammals but in some species follicular development, in the absence of the pituitary, does not progress as far as it does in the rat (see Smith, 1939, for references).

The absence of pituitary control over oogenesis and early development of the

follicle is probably characteristic of all vertebrates. Smith (1939) found that hypophysectomy of frog larvae (*R. boylei*) did not prevent growth of the gonads up to the stage normally attained at the time of metamorphosis. Similar observations have been made on other species of amphibia (Burns, 1932; Atwell, 1933; and Witschi, 1937) and Shapiro and Shapiro (1934) report not only the degeneration of large ova in toads (*Xenopus laevis*) but also an increase in the number of small follicles.

We know almost nothing in this regard about reptiles and only a few observations have been made on birds. Hill and Parkes (1934) found no follicles of a greater diameter than 1 or 2 mm. in the ovary of a hypophysectomized hen and state that the condition produced is essentially similar to that found in the non-laying bird at the height of the moult. Probably the most important difference found among the vertebrates is the size the ovum can attain after ablation of the hypophysis. The mammalian ovum can reach full size but the growth of large ova in other groups is limited by an inability to deposit yolk.

The sequence of events seen in the formation of primordial follicles in the mammalian ovary is suggestive of developmental processes observed in the organization of tissues in an embryo. The similarity is so striking that one is tempted to postulate that it is probably brought about through the co-ordinated interaction of a self-contained system of organizers within the growing follicle. The actual existence or nature of such organizers in the follicle has not been demonstrated, but organizers are known to be present in the developing gonad (Witschi, 1939). The arrangement of the granulosa of a primordial follicle is apparently under the control of the ovum. When the ova are destroyed in mice and rats by x-ray treatment the germinal epithelium may form cords and clumps of granulosa cells instead of follicles (Parkes, 1926, 1927; Brambell, Fielding and Parkes, 1928; Drips and Ford, 1932; Allen, Hisaw and Gardner, 1939). Although there is much confusion concerning the details of the effects of x-rays on the ovary and the histogenesis of tissues following a treatment, yet it seems that aggregates of granulosa cells can organize the connective tissue of the stroma into structures resembling a theca interna. If this is true, then it is clearly the granulosa and not the ovum that is responsible for the formation of the theca. At the same time the theca interna provides the follicle with a blood supply and either this or a stimulus derived from the theca itself may account for the increase in mitotic activity of the granulosa in a growing follicle.

It has been shown that estrogen has a direct effect on the ovary and this is of additional importance since there is considerable evidence that the theca interna is at least one of the sites of formation of estrogen (Corner, 1938; Mossman, 1937). Bullough (1942) found that intraperitoneal injections of estrogen greatly increased mitotic activity and oogenesis in the germinal epithelium in such distantly related vertebrates as mice and minnows (*Phoxinus laevis*). He suggests that the increased mitosis in the granulosa of a growing follicle might also be due to estrogen. It is interesting that estrogen can act as an organizer at its source of formation, and also function in the co-ordination of more remote bodily processes.

Regardless of the mechanism follicular growth can proceed in mammals, as

has been mentioned, up to the appearance of the antrum folliculi in the absence of the pituitary. This condition is reached in the normal mouse by the twelfth to fourteenth day of age (Engle, 1931) and in rats by the eleventh day (Hargitt, 1930). Previous to this time, the young follicles in both rats and mice are refractory to the gonadotropic action of pituitary hormones. A similar situation exists in rabbits (Hertz and Hisaw, 1934). Probably the ovaries of all mammals are refractory to gonadotropins from birth to a specific age which is characteristic of each species. Clauberg (1932) speaks of this period as "infantile" and the interval between the appearance of responsiveness and the first estrus as "juvenile." The morphological difference between a follicle that will not respond to pituitary hormones and one that has gained competence to react is not obvious. However, it seems that the acquirement of sensitivity is correlated with the time at which the cells of the theca interna normally start differentiating into epithelioid tissue.

Another indication that a follicle has emerged from the "infantile" into the "juvenile" condition, and consequently is susceptible to pituitary stimulation, is the presence of an antrum. The nature of the conditions under which antra are formed is not fully understood but the results of experiments by Lane (1935) are suggestive. Lane first made a statistical study of the ovaries of normal rats from 15 days of age to sexual maturity and of rats hypophysectomized at 28 days of age and killed from one to 38 days later. The larger follicles were classified into two groups; those having two or more layers of granulosa cells were called primary follicles and those having antra were designated as vesicular follicles. These data were used in determining the effects of pituitary hormones and estrogen on the ratio between these two classes of follicles and their total number.

Lane found that 22 day old rats given FSH twice daily for three days and killed when 28 days of age showed an increase over normal in the total number of primary and vesicular follicles, but the percentage of vesicular follicles remained about the same. When LH was used the total follicular number varied little from that of the untreated animal but there was a marked increase in the percentage of those follicles having antra. These gonadotropic preparations also produced similar modifications in the ratio between primary and vesicular follicles in hypophysectomized rats (Lane and Greep, 1935). These results indicate that the formation of follicular antra in rats is associated with the action of LH. It is quite probable that LH plays an important part in this process but whether it alone is directly responsible for producing this effect has not been satisfactorily proven.

It also seems possible that estrogen may influence follicular development by causing an increased secretion or release of LH from the pituitary. Hohlweg (1934) and Hohlweg and Chamarro (1937) found that luteinization could be produced in the ovaries of immature rats by injecting estrogen. Lane (1935) also found that an estrogen treatment caused an increase in the percentage of antra containing follicles, in the ovaries of immature rats, similar to that observed following the administration of LH. Other observations that tend to support the opinion that estrogen stimulates the secretion of LH by the pituitary will be presented in the subsequent discussion of ovulation.

The immediate point under discussion concerns the factor or factors that enable a young follicle to gain competence to respond to the gonadotropic hormones of the pituitary and thus start on its way toward full maturity and ovulation. There are very good reasons for assuming that FSH, LH and estrogen are present at the time the ovary of a young animal gains competence to respond to injected pituitary gonadotropins. Yet we are quite ignorant concerning the individual rôles played by these hormones in the conditioning processes in a young follicle. It is evident that the earlier experiments just cited, though important and suggestive, fall short of the mark in that they do not establish the individual action of a particular hormone to the exclusion of all others. The pituitary gonadotropic preparations were admittedly not pure and that FSH and LH were separated completely is unlikely. Recently FSH and LH have been isolated as pure proteins and should prove to be of great value in the solution of this problem. (For references see Chow, 1943, and Fevold, 1943.)

What seems to be needed are experiments in which the separate and combined actions of these gonadotropins, and estrogen, are tested on the ovaries of hypophysectomized animals. Some very important investigations of this nature have been made but so far more emphasis has been placed on changes in ovarian weights and gross morphology than on the follicular populations and cytological modifications of the ovary.

Greep, van Dyke and Chow (1942) studied the effects of LH (metakentrin, ICSH) and FSH (thylakentrin) isolated as pure proteins from hog pituitaries, on the ovaries of hypophysectomized immature rats. The LH preparation prevented atrophy in the interstitial cells when the treatment was started soon after the operation or would repair the atrophic changes when the treatment was begun several days post-operatively. It also produced enlargement of the thecal cells and at high doses converted some of the smaller follicles into pseudo-lutein bodies. These authors made little point of the presence of antra other than to mention their absence in the healthy-appearing follicles in control animals killed twelve days after hypophysectomy and the presence of microscopic antra in a few follicles in the ovaries of animals that had received LH.

The administration of pure FSH caused the Graafian follicles to grow, mature, and pass into atresia without becoming luteinized or cystic. The ovaries remained small even in animals given large doses and the follicles did not attain the size of mature follicles in normal rats. The interstitial tissue and theca interna were not affected and there was no indication of estrogen secretion. Results that agree with these in most respects have been reported for pure gonadotropins obtained from sheep pituitaries (Fraenkel-Conrat, Li and Simpson, 1943).

When hypophysectomized rats were given a combination of pure FSH and LH simultaneously the effects on the ovaries were like those produced by unfractionated pituitary extracts (Greep, van Dyke and Chow, 1942). The ovaries were heavier than could be obtained with either hormone alone. The follicles grew to full size and were luteinized while the theca interna was well developed and estrogen was secreted. These experiments confirm previous ideas as to the functions of the pituitary gonadotropins in follicular development (Fevold, 1939)

but they leave many questions unanswered and at the same time raise a new one concerning the possible importance of the direct action of estrogen on the follicle.

Williams (1940, 1944, 1945) recently has made the observation that tablets of diethylstilbestrol implanted subcutaneously in immature female rats two days after hypophysectomy prevent or greatly retard the atrophy of the ovaries. He also found that the response of such animals to the injection of pregnant mare serum was markedly increased and the luteinization of the granulosa was much more pronounced than in the nonestrogenized controls. Pencharz (1940) also has reported an increase in size of the ovaries of hypophysectomized rats given subcutaneous implants of diethylstilbestrol. The increase in ovarian weight in response to diethylstilbestrol was about three times that produced by estradiol dipropionate while testosterone propionate was without effect. It was suggested that this discrepancy between the action of the two estrogens probably was due to a difference in rate of absorption.

These estrogens, and especially diethylstilbestrol, had a marked effect on the follicles. The ovaries of the treated animals were packed tightly with medium-sized follicles and the interstitial tissue was greatly reduced. Pencharz also made the interesting observation that the reaction to estrogen gave the otherwise refractory ovaries of the hypophysectomized animal a most remarkable ability to respond to chorionic gonadotropin.

Simpson, Evans, Fraenkel-Conrat and Li (1941) confirmed the work by Pencharz and also studied the effects of estrogens on the response of the ovaries to FSH and LH preparations. They noted that the increase in size of the ovaries of hypophysectomized rats given estrogen was due to a "slight but definite" growth of many follicles. Such follicles in spite of an increase in size did not develop antra and it is mentioned that some showed a peripheral zone of degeneration. This last point was not discussed in detail but it appears that the defect (ref. fig. 4) was in the basal part of the granulosa. The estrogens did not repair the interstitial tissue and presumably this was also true for the theca interna although it was not described.

The influence of diethylstilbestrol on the action of LH and FSH was tested on immature female rats that were hypophysectomized at 28 days of age. Estrogen treatment was started the day after hypophysectomy, the gonadotropins were given on the fifth to seventh days and the animals were killed on the ninth. The estrogen did not modify the action of LH, the reaction being the additive effects of both hormones. However, there was a marked increase in the reaction of the ovaries to FSH. FSH alone produced development of large and medium follicles and there was no repair of the interstitial tissue. When the same dosage of FSH was given to animals that had received diethylstilbestrol the ovaries attained a much greater weight and contained large follicles, corpora lutea and thecal luteinized follicles while the interstitial tissue "was not repaired (or only partly)." The luteinization was probably due to the presence of LH as Fraenkel-Conrat et al. (1943) state that they had not been successful in eliminating all of the luteinizing hormone (ICSH) from their FSH preparations. However,

these results indicate that estrogens greatly potentiate the action of FSH in the hypophysectomized animal and at the same time, judging from dosage, suggest the possibility that the treatment made the theca interna and interstitial tissue more sensitive to the action of LH.

These observations on sexually immature animals suggest several points of interest that can be discussed better after follicular growth in the estrous cycle of the adult has been considered.

In the normal estrous cycle of guinea pigs and rats those follicles that are destined to ovulate begin their development during a period of estrus and reach maturity at the succeeding estrus. During the intervening diestrum they maintain a growth-rate that is nearly constant (Dempsey, 1937; Boling, Blandau, Soderwall and Young, 1941). This probably holds for most mammals in which the estrous periods follow each other in more or less rapid succession and as far as the history of an individual follicle is concerned, after growth has started, this seems true in a general way for all vertebrates. The chief difference being a matter of time.

Some very significant facts regarding follicular growth have been obtained by examining the mitotic rate in the granulosa and theca through the use of colchicine. Schmidt (1942) made a thorough study of the follicles in the ovaries of guinea pigs, from the time of their origin in the germinal epithelium (Schmidt and Hoffman, 1941) to ovulation and the formation of corpora lutea. Primary follicles 25 to 30 μ in diameter are formed from cell nests in which the flattened, undifferentiated granulosa cells become arranged around the young ovum. This process seems to take place without mitosis and mitotic figures are rarely seen in follicles less than 60 μ in diameter, at which time the flattened granulosa cells are differentiating into the typical cuboidal type.

In the guinea pig the follicles acquire a thin theca interna as they approach a diameter of 200 μ and at this stage, or soon afterwards, a small antrum is formed. At this point, as indicated by mitotic activity, the follicle enters a period of active growth, presumably under pituitary influence. At first this is slow but becomes rapid when the follicle has reached a diameter of about 300 μ. The growth-rate in both granulosa and theca is most active from this time until the follicle is 600 μ in diameter. This is followed by a gradual decline in mitotic activity in the theca while in the granulosa the number of mitoses continues to increase, though growth is no longer as rapid, until a follicular diameter of 800 μ is attained. Thus it is seen that the period of rapid mitoses in the granulosa is preceded by the formation of a theca interna and when mitotic activity reaches its peak in the theca and begins to decline this change apparently is reflected in the granulosa by a decrease in mitotic rate.

These peaks of mitotic activity doubtless indicate an abrupt turning point in the physiology of the follicle. Those that escape atresia continue to increase in size, primarily through the accumulation of follicular fluid, but the number of mitoses rapidly decreases and they are absent at ovulation. Follicles having a diameter greater than 800 μ are first seen on the eleventh day of the cycle. This is very significant, as the eleventh day is also the average time that guinea pigs

come into estrus when the cycle is shortened by removing the corpora lutea after ovulation (Loeb, 1911; Dempsey, 1937). Also Dempsey, Hertz and Young (1936) found that ovulation could be induced in the guinea pig as early as the twelfth day of the cycle by injecting an LH preparation. Therefore, the follicle becomes "mature," in the sense that it is capable of ovulation, soon after its diameter increases beyond 800 μ. However, in the normal cycle the sudden spurt in growth, or preovulatory enlargement, may not occur until three or four days later.

These observations on the guinea pig probably represent the general situation in mammals. The only other animal that has been studied with an equal degree of thoroughness is the rat. Lane and Davis (1939) employed the colchicine technique in a careful study of rat ovaries selected at various times during the estrous cycle. Their results differ from those mentioned for the guinea pig only in matters of degree in that the events seen in the sixteen-day cycle of the guinea pig occur more rapidly in the four to six-day cycle of the rat (Boling, Blandau, Soderwall and Young, 1941).

It is obvious that the facts at hand are inadequate as a basis for definite conclusions concerning the physiological mechanism directing and co-ordinating the formation of a follicle, yet they present certain suggestive features that encourage speculation. In the first place it seems probable that the follicle is under two sets of controls, one within the ovary itself and the other outside. The endogenous factors in the ovary work on the principle of embryological organizers. For example, the primordial ovum seems able to arrange the cells that surround it into a granulosa one cell in thickness. The infrequent mitoses in such follicles indicates that the growing ovum probably exercises little if any influence on such activity. However, the fact that during atresia and preovulatory enlargement preceding ovulation, the last place in which mitotic activity disappears is the germ hill, suggests that the mature ovum possesses some ability to stimulate mitoses.

The organizing action of the ovum also is indicated by the observation that after the egg is killed by x-rays, the granulosa cells remain in clumps and cords. Also, the fact that the granulosa can persist in the absence of an ovum makes possible further deductions. Ovaries of x-rayed animals secrete estrogen and if the theca interna is the source of estrogen we may conclude that it is the granulosa rather than the ovum that is responsible for organization of the theca. However, what influence if any progesterone might have on the organization of a follicle in the estrous cycle of mammals is as yet unknown.

The active agents formed outside the ovary are the pituitary gonadotropic hormones FSH and LH which, by their combined action, cause the secretion of estrogen within the ovary. The results of the investigations that have been cited definitely show that from the time a follicle enters the period of rapid growth until ovulation it is constantly under the influence of these three hormones. Furthermore, these observations also give some indication of the function of each hormone. Maintenance and maturation of the theca interna are controlled by LH. Also, FSH cannot promote the secretion of estrogen in the

absence of LH. It seems probable, though it has not been proven, that the theca interna through the action of LH acquires competence to respond to FSH.

One of the functions of FSH is to produce enlargement of the follicle, primarily by the accumulation of fluid in the antrum. This action is limited in the hypophysectomized rat as shown by the failure of the follicles to reach normal size (Greep, van Dyke and Chow, 1942). The reason for this is not clear nor has the source of the follicular fluid been determined. Neither is it known with certainty which structure, if any, in the follicle gives a specific response to FSH. It has been generally thought that the granulosa was the structure concerned but this probably should be questioned now since it is known that hyperplasia of the granulosa can be induced with estrogens and that this effect greatly increases the response to FSH. This, of course, raises the question as to the importance of estrogen, which is always present, during the simultaneous action of FSH and LH.

A point worth considering is the possibility that estrogen secreted by the theca interna in response to FSH and LH may function in follicular development as the growth stimulating agent for the granulosa. This thought is not invalidated by the fact that estrogens must be administered in large doses to produce this effect (Williams, 1944). The theca interna closely invests the granulosa and the constant secretion of minute amounts of estrogen would be many times more effective under such a situation than much larger amounts given subcutaneously. Also, the fact that estrogen is secreted in the ovary may be a good physiological reason for a much higher threshold for ovarian structures, if such exists, than for responsive tissue elsewhere in the body.

Another possibility worth mentioning before concluding this line of thought is that some estrogen may be secreted in the ovaries of very young or hypophysectomized animals. The presence of lipoidal material in the thin theca of small follicles less than 200 μ in diameter, as demonstrated by histochemical techniques, lends support to this idea (Dempsey and Bassett, 1943). Follicles in the rat's ovary do not respond to pituitary gonadotropins before they have a diameter of about 200 μ and Williams finds that the granulosa of primordial follicles is not stimulated by estrogens. It is also true that mitosis in such follicles normally proceeds at a relatively slow rate. These observations make one wonder if the granulosa gains competence for rapid growth in response to estrogens at the same time that the follicle becomes sensitive to gonadotropins. If this is true, then it seems possible that the pituitary gonadotropins, for one thing, simply take over and augment a process that already has been started.

A question that often is raised is why a juvenile animal whose ovaries are capable of being stimulated by injected gonadotropins, does not come into sexual precocity in response to her own pituitary secretion. The histology of the ovaries and the changes that occur following pituitary ablation clearly show that the pituitaries of such animals are actively secreting. It is also true that the ovaries may show several cycles of follicular growth and atresia before the first estrus.

We have no quantitative data that are satisfactory for an analysis of these

facts. Several investigators have mentioned the possibility of an increase in secretion of the gonadotropins as the animal approaches maturity. There are no acceptable techniques for determining the exact amount of FSH and LH in pituitary tissue or unfractionated extracts (Fevold, 1939) and until such are devised this point cannot be settled. Another possibility worth considering is that the follicles may become more responsive to a given amount of pituitary gonadotropins with the advance in age of the animal. It has been shown that this is true for the reactivity of the male and female accessory reproductive organs to estrogens and androgens (Hooker, 1942; Price and Ortiz, 1944; Price, 1944).

We know considerable about the physiology of follicular development but there is much yet to be learned about specific processes involved. One of the obstacles that has retarded advancement has been the lack of pure preparations of pituitary gonadotropins but this now has been removed. Pure LH has been prepared in three laboratories (Chow, van Dyke and Greep, 1942; Li, Simpson and Evans, 1942; Fevold, 1943) and FSH in one (Chow, 1943). It is true that so far the reactions produced in the ovary by these pure substances tend to confirm previous reports for FSH and LH preparations, in which the two hormones were not completely separated. However, pure FSH and LH have not been used in making a critical analysis of follicular development and their effects on the follicular populations of the ovary should be determined. This would contribute to a better understanding of the synergistic reaction produced in the ovary by the action of the two hormones. It has been demonstrated that growth of the granulosa in response to estrogen facilitates the response of the follicle to FSH, but it has not been shown that the changes produced in the theca interna by pure LH will also potentiate FSH. All that is known, in this respect, for the pure gonadotropins is that the response of the ovary is greater when FSH and LH are given simultaneously; however this does not eliminate the participation of estrogen in the reaction. A detailed study of morphological changes in the follicle induced by the independent and concurrent actions of the pure gonadotropins and estrogens would greatly advance our knowledge of ovarian physiology.

Ovulation. The rupture of a mature Graafian follicle and release of an ovum has been the subject of more research during the last twenty years than almost any other problem concerned with ovarian physiology. Out of this have come certain well established facts, one of which is that in normal animals ovulation is under the control of the pituitary. Also, the reaction can be induced by injecting the gonadotropins obtained from the urine of pregnant women and the blood serum of pregnant mares.

This review is concerned with development of the follicle and ovulation in the normal estrous cycle; therefore we are interested in pituitary-ovarian interactions. However, experiments in which the chorionic gonadotropins were employed have contributed much toward a better understanding of normal ovulation, and are useful for our discussion. Yet, one should keep in mind the fact that chorionic gonadotropins are hormones of pregnancy and are neither chem-

ically nor physiologically identical either with each other or with pituitary gonadotropins. Their gonadotropic action, though interesting and useful for experimentation, is nevertheless incidental to their normal function (Engle, 1939; Astwood and Greep, 1939; Hisaw, 1944).

The rupture of a follicle, as seen in mammals, is preceded by a period of rapid growth referred to as pre-ovulatory enlargement. A similar phenomenon probably occurs in other vertebrates but is modified and obscured by the presence of large yolk-laden ova. The morphological changes that take place during pre-ovulatory enlargement and rupture of the follicle have been described in detail for several species. The literature dealing with this has been reviewed by Hartman (1939) and the reader is referred to this and a more recent paper by Dawson (1941).

The follicles that undergo pre-ovulatory enlargement and ovulation in the estrous cycles of guinea pigs and rats are those that have survived a period of extensive atresia (Schmidt, 1942; Lane and Davis, 1939). Such follicles can be distinguished by the third day of the cycle in the rat and by the twelfth day in the guinea pig. From this time until pre-ovulatory enlargement sets in there is a progressive increase in diameter and a further differentiation in the theca and granulosa. The most conspicuous change seems to involve the theca and consists of an increase in blood supply, shown by a rapid proliferation of endothelial cells which becomes enormous during pre-ovulatory enlargement.

The number of eggs ovulated at one time is quite constant for the different species which raises the question as to why certain follicles are selected and others are eliminated by atresia. There is no obvious answer but it seems possible that the immediate cause of atresia might result from a failure of proper differentiation of the theca interna. This thought seems more plausible when one considers the differential action of FSH and LH on the ovary. Fevold (1937) found that the ovaries of hypophysectomized rats showed a greater response to an FSH preparation when the treatment was preceded by three daily injections of LH. Although the LH preparation did not cause an increase in ovarian weight, the effects it produced augmented the response to FSH. The gross appearance of the ovaries indicated that the reaction to FSH was facilitated by an increase in the number of follicles that were capable of responding.

There are a number of facts that tend to support the idea that atresia is due to defective differentiation of the theca interna resulting from inadequate stimulation by LH. The first of course is the specific action of LH on the theca interna resulting in hypertrophy of the thecal cells and increase in blood supply. Another is the theca as a source of estrogen (Corner, 1938) and the part it might take in the growth and differentiation of the granulosa. If estrogen is responsible for growth of the granulosa then the observation that indications of atresia first appear in that structure also suggests failure of the theca (Mossman, 1937; Schmidt, 1942).

The fact that atrophy of the follicles in hypophysectomized rats is prevented by the direct action of injected estrogen on the granulosa also supports the argument (Williams, 1945). The effect of estrogen on the ovary resembles that of

LH in that it apparently increases the number of follicles that are responsive to FSH, yet it differs in a very important respect. Although LH alone prevents atrophy of the theca and promotes its differentiation it cannot maintain the follicles for an extended period, the probable reason being that estrogen is not secreted. It is logical to infer, from experimental evidence, that the well-being of a follicle depends upon the co-ordinated action of FSH, LH, and estrogen. A recent contribution to this thought is the observation by Williams (1940) that estrogen increases the sensitivity of the granulosa to the luteinizing action of pregnant mare serum. Whether or not this explanation for atresia of large follicles previous to ovulation is entirely correct, it seems evident that such follicles do not become atretic necessarily because they are incapable of responding to developmental stimuli.

Superovulation. That many more follicles are capable of being brought to ovulation than normally occurs has been known since the early experiments of Smith and Engle (1927). They obtained 20 to 48 ovulations in rats and mice by implanting pituitary tissue. Such ova were capable of being fertilized and as many as 29 implantations were found on the ninth to tenth day of pregnancy (Engle, 1927, 1931). Later, Cole (1937) obtained similar results with pregnant mare serum. Superovulation has been induced in several species by injecting gonadotropic preparations, but for the purpose of this discussion we are interested primarily in quantitative data that show the conditions under which the response can occur and the nature of the reaction. Unfortunately such papers are not many and data are difficult to compare due to differences in method of preparation and standardization of gonadotropic extracts. This is particularly true of pituitary gonadotropins for which there is no international unit.

Evans and Simpson (1940) used a highly purified FSH preparation in experiments on superfecundity in rats, as indicated by supernumerary implantation. This material was used on female rats 26 to 34 days of age that were placed with males 48 hours after the beginning of treatment. A total dose of 10 to 15 RU gave the highest number of implantation sites, while higher doses were progressively less successful and low doses inadequate. Almost all the animals that breed did so between 48 and 60 hours after the onset of treatment. These results are in agreement with those of Engle (pituitary implants) and Cole (PMS) and show that in both mice and rats approximately 30 implantations can be produced and the number of live young born (17) increased about three times the normal number.

Rowlands (1944) reports that 50 to 60 gram rats given single injections of 2 to 60 IU of pregnant mare serum did not give consistent results either in number of ova ovulated or number of animals responding. However, in a second series of experiments it was found that ovulation could be produced regularly in such animals if the serum treatment was followed by an injection of pregnancy urine hormone. The optimal interval between injection of serum gonadotropin and pregnancy urine hormone was 48 to 72 hours, with the best results at 56 hours. It is also interesting that the number of ova ovulated was correlated with the dosage of serum gonadotropin and not with the amount of pregnancy urine

hormone used to induce ovulation. Superovulation could be produced following the injection of 30 IU of serum gonadotropin but almost no ovulation could be obtained after a dose of 60 IU.

Williams (1945) has shown that a single injection of serum gonadotropin will produce ovulation in hypophysectomized rats until the fourth day following the operation, but not later. That the failure after the fourth day was due at least in part to ovarian atrophy was demonstrated by injecting serum gonadotropin followed by an injection of sheep or pig gonadotropin, pregnancy urine hormone, or a second treatment of pregnant mare serum (Rowlands and Williams, 1943). The optimum interval between treatments was found to be four days and fewer animals responded when the second treatment was postponed until the fiftieth day by which time follicular atrophy may have started.

If ovarian atrophy following hypophysectomy was prevented by implanting tablets of stilbestrol ovulation could be induced by a single injection of serum gonadotropin as in a normal animal (Williams, 1945).

Rabbits also have been used in investigations on superovulation. Pincus (1940) has reported extensive experiments on prepubertal and pubertal rabbits five or six months old and mostly of a chinchilla strain. His usual procedure was to stimulate follicular development by giving six subcutaneous injections of a gonadotropin over three days followed within eight to twelve hours by an intravenous injection to induce ovulation. The preparations used to promote the growth of follicles were pregnant mare serum, FSH fractions of sheep pituitaries, and horse pituitary extract. Those employed for intravenous injections were the serum gonadotropin, horse pituitary extract and a combination of FSH and LH extracts of sheep pituitaries. He found that all of these gonadotropic preparations would produce ovulation when properly administered but the pituitary extracts usually caused superovulation while mare serum would only occasionally do so. As many as 80 ova were obtained from a single rabbit. He also found that in these young animals copulation could not be substituted for the intravenous injection for inducing ovulation.

Parkes (1943) produced superovulation and superfecundation in adult rabbits by "priming" them with an extract of horse pituitaries and producing ovulation by an intravenous injection of pituitary extract or pregnancy urine hormone, or by mating. In experiments to determine the most favorable dosage and length of treatment for the growth of follicles in rabbits, he found 1 or 2 mgm. of the pituitary extract daily better than 5 mgm. and a five-day treatment better than three. The larger dosage produced cystic and hemorrhagic follicles. He also found that sexually immature Lop rabbits weighing about 2.0 kgm. would mate and become pregnant following a priming treatment. The ovaries of those rabbits weighing approximately 1.0 kgm. were insensitive to gonadotropins while animals between 1.0 and 2.0 kgm. showed two different conditions; the follicles responded to priming but did not ovulate after mating or ovulation took place but corpora lutea did not develop.

These investigations in which gonadotropins have been used to promote follicular development and ovulation, have a number of points in common. In

growing follicles with gonadotropins, there is both an optimal dosage and an optimal length of treatment. Comparisons of dosages for the different preparations are difficult to make, for reasons already mentioned, but the time required for the response is approximately the same for all. From the beginning of treatment, mating in rats occurs between 48 and 60 hours (Evans and Simpson, 1940); optimal time for inducing ovulation with pregnancy urine hormone in normal rats, 48 to 72 hours (best at 56 hrs.) and in hypophysectomized rats, four days (Rowlands, 1944; Rowlands and Williams, 1943). In rabbits, Pincus (1940) found three days as the optimal length of treatment for the immature animal while Parkes (1943) found five days better than three for the adult, both as to the number of ova ovulated and the number of large follicles in the ovaries. This is probably significant for the adult animal as Hill and White (1933) in a study of regression of follicles in the estrous rabbit noticed that a decrease in size and disappearance of follicles started on the fifth day.

These observations indicate that a follicle grows at a definite rate and the time required to reach a condition susceptible to ovulatory stimulation cannot be shortened greatly by increasing the growth stimulus. As the dosage of a gonadotropin is increased more and more follicles become involved in the growth process until a point is reached at which defects appear in the form of cystic follicles and premature luteinization. When pituitary extracts are used, such abnormalities may be due to the presence of LH either in the preparation or from the animal's own pituitary. Such defects are not found even when large doses of pure FSH are given to hypophysectomized rats (Greep, van Dyke and Chow, 1942). The luteinizing property of pregnant mare serum and pregnancy urine hormone also gives rise to a similar situation when they are employed as growth-promoting agents in follicular development.

Another point of interest is that the number of follicles that can be brought to ovulation by the priming process greatly exceeds the number of large follicles from which selection is made in the normal cycle prior to preovulatory enlargement. It seems obvious that a large number is included from those that had not arrived at the point of differentiation before the treatment was started. This does not imply necessarily that follicular growth rate was increased as it seems more probable that differentiation of the theca and granulosa was brought about before the follicle reached full size. This supposition has some support in the fact that ovulation can be induced before a follicle attains its full diameter (Dempsey, 1937).

Ovulation in sheep and cattle. The application of gonadotropins to problems in animal husbandry has contributed much information concerning ovulation in farm animals. Most of this work has been done in Russia, Great Britain, and the United States and the reader is referred to recent papers by Hammond (1945); Hammond and Bhattacharya (1943); and Cole, Hart and Miller (1945) for references to the literature. From the standpoint of this review the most useful data were obtained from studies of ovulation in sheep and cattle. Those for cattle are the most important due to the advantage that conditions in the ovaries can be determined by manual palpation per rectum (Hammond, 1927).

Cole and Miller (1933) showed that ovulation could be induced in anestrous sheep by injecting serum gonadotropin but the results were highly variable and not usually accompanied by estrus. Hammond (1945) mentions that the absence of estrus in such experiments is like the first ovulation of the natural breeding season and may be followed, by the length of an estrous cycle, by spontaneous ovulation and estrus. Also, a second treatment following the first by about sixteen days would cause ovulation and estrus. Probably the most suggestive observation by Hammond was that ovulation could be produced by injecting stilbestrol or stilbestrol di-n-butyrate. Seven out of eleven animals given 1 to 5 mgm. stilbestrol did not ovulate but ovulation occurred in five of six treated with 0.25 or 0.5 mgm. of stilbestrol di-n-butyrate. It also was found that stilbestrol tends to inhibit ovulation if given simultaneously or one or two days before serum gonadotropin, but when the estrogen was injected twelve to twenty-four hours after the serum, there was no inhibition or it was of a lesser degree. Another observation was, that the ovulating action of both stilbestrol and serum gonadotropin generally was inhibited by the presence of a functional corpus luteum.

Several investigators have produced ovulation and multiple pregnancies in cattle but the most recent and, in many respects, the most detailed study is that by Hammond and Bhattacharya (1943). Most of their experiments were performed on cows and heifers that had been selected for slaughter while a few were bred and left to calve. They succeeded in producing superovulation and multiple pregnancies but the object of their investigation was a practical one, the production of twin ovulations. For this purpose they concluded, from preliminary experiments, that 1500 IU of serum gonadotropin or about 100 mgm. of horse pituitary extract was the most favorable dosage to use for stimulating follicular growth. However, before discussing this point the influence of a functional corpus luteum on ovulation should be mentioned.

It is now well known that if a functional corpus luteum is expressed from the ovary of a cow, estrus and ovulation follow within about four days. The corpus luteum apparently does not prevent follicular growth in response to a gonadotropin but it does inhibit ovulation. This seems particularly true for reasonably small doses (1500 IU) but heavy dosage (5000 IU) may produce ovulation in the presence of the corpus luteum. Hammond and Bhattacharya took advantage of these facts and most of their experiments deal with the relation between dosage of gonadotropin and the time of removal of the corpus luteum. Various amounts of serum gonadotropin and horse pituitary extract were given, in most instances, from five days before to two days after the corpus luteum was removed. However, for present purposes it seems sufficient to mention the results obtained when 1500 IU of serum gonadotropin was used. Although these results were quite variable it seems that the most effective time for administering this dosage for producing approximately two ovulations was two days before to the day after corpus luteum removal.

These observations on sheep and cattle show that progesterone and estrogen have an important influence on ovulation. The presence of a functional corpus

luteum (presumably progesterone) inhibits ovulation. In sheep, estrogen seems to have two effects one of which depends upon the time it is injected in relation to the administration of a gonadotropin. Estrogen alone may induce ovulation if a large follicle is present and if injected before a gonadotropin may inhibit the ovulation that ordinarily occurs.

Influence of estrogen and progesterone on ovulation. Observations similar to those reported for sheep and cattle have been made on laboratory animals. From these studies it seems that ovulation produced by the injection of estrogen is not due to a direct action on the ovary but probably results from an effect on the pituitary causing an increased output of LH. It has been shown in rats that the secretion of LH by the pituitary can be stimulated by injecting estrogen (see Fevold, 1939, for references). A three-day treatment with FSH causes only follicular development in the ovaries of immature rats, but if preceded by three daily injections of estrogen both luteinization and augmentation of ovarian weight takes place. However, if hypophysectomized animals are used the estrogen treatment does not modify the response to FSH, thus showing that the LH came from the pituitary as a result of the action of estrogen.

The inhibitory action of estrogen on the response to serum gonadotropin in sheep also has its counterpart in experiments on rats (Fevold and Fisk, 1939). While a three-day treatment with estrogen will augment the response of the ovary to FSH, an eight-day treatment prior to the injection of the gonadotropin will greatly decrease the response. Fevold and Fisk found that rats on estrogen for eight days had a higher concentration of LH in the blood than those treated for three days. They also showed that the same results could be obtained by injecting an LH preparation, and demonstrated, in both normal and hypophysectomized animals, that the extent of inhibition was correlated with dosage of LH.

Thus it seems that while a small amount of LH augments the action of FSH the presence of a large amount, or effects produced by prior action, inhibits the response of the follicle to a follicle-stimulating gonadotropin. Yet, there is considerable uncertainty as to whether such inhibition is the result of a direct action of LH on the follicle or is brought about by some other means (Fraenkel-Conrat et al. 1940ab.; van Dyke, 1939). However, it is significant that pure LH will inhibit the gonadotropic action of pregnant mare serum on the ovaries of immature rats (Greep, van Dyke and Chow, 1942).

The inhibitory influence on ovulation exercised by the mammalian corpus luteum apparently is accomplished by the secretion of progesterone. It has been mentioned that a functional corpus luteum does not inhibit follicular growth but prevents preovulatory enlargement and rupture of the follicle. This is in line with experimental results indicating that progesterone inhibits secretion of LH by the pituitary. Makepeace, Weinstein and Friedman (1937) found that estrous rabbits under the influence of progesterone did not ovulate when mated. Astwood and Fevold (1939) likewise have shown that pseudopregnancy in rats, which usually follows electrical stimulation of the uterine cervix, did not occur if progesterone was injected at the time or prior to the application of

the stimulus. Progesterone also will prevent the action of estrogen on the pituitary resulting in the release of LH (Astwood and Fevold, 1939). It is a common observation that immature rats on a prolonged treatment with FSH show first follicular development followed by luteinization. Such luteinization is thought to be due to LH secreted by the pituitary in response to estrogen from the stimulated follicles. If, in such experiments, progesterone is also given the follicles are not luteinized. Therefore, it seems that when a gonadotropin which itself has no or only weak luteinizing capacity is used to grow follicles in the presence of a corpus luteum, as in cattle, ovulation does not take place because the luteinizing action of the injected hormone is insufficient or lacking and progesterone secreted by the corpus luteum inhibits release of LH from the pituitary. In this connection it is interesting to note that Hammond and Bhattacharya (1943) obtained no ovulation with 1500 IU of pregnant mare serum in ten cows having corpora lutea but of four others given 5000 IU one had a single ovulation.

There are, however, other facts that tend to suggest that progesterone may influence ovulation in ways other than those just mentioned. One of these is brought out by Hammond (1939) in a discussion on the treatment of cystic follicles in cattle. He found that after a cyst is ruptured another follicle develops but does not ovulate, but if this one is ruptured it luteinizes and is followed by a normal cycle and estrus three weeks later. Day (quoted by Hammond and Bhattacharya, 1943) found that a normal cycle may also be brought about by rupturing a cyst and administering pregnancy urine extract. These observations indicate that a period of luteal activity in some way restores the hormonal balance requisite for ovulation.

A very interesting series of studies of a similar nature was started by Everett in 1939 on a strain of rats that has a tendency to remain in constant estrus. This condition, once it appears, persists indefinitely except for short diestrous periods at infrequent intervals. The ovarian follicles are slightly cystic, being larger than normal, and the nuclei of the interstitial tissue present varying degrees of achromatolysis and "wheel" figures resembling the condition seen in hypophysectomized rats. These defects suggest a deficiency in LH while in the case of the follicles the mild overgrowth probably indicates very weak LH action.

Everett has found it possible to correct this situation and restore normal ovulation and luteinization by administering progesterone. In one series of experiments (Everett, 1940) he gave persistent estrous rats daily injections of progesterone in doses below that required to inhibit the estrous cycle of a normal animal. Several rats were kept on this treatment for 40 to 50 days and maintained fairly regular cycles, but all returned to constant estrus when the injections were stopped. Another method (Everett, 1943) was to give a single "interrupting" dose of 0.5 to 1 mgm. of progesterone which usually caused atresia of the large follicles and the appearance of diestrus lasting for two or three days, during which a new set of follicles grew. If the treatment was concluded at this point 90 to 95 per cent of the animals would return to persistent estrus following the brief diestrous interval. However, ovulation and luteinization could be induced

by a second injection if given at proper intervals after the first. The effectiveness of the second injection was slight in early diestrus, rose to a maximum on the first day of the succeeding estrus, and was negligible after estrus had persisted longer than eight days. Therefore, on the basis of these facts and the results of other similar studies (Everett, 1944a, b; 1945) Everett concluded that in rats small amounts of progesterone cause the release of LH from the pituitary.

Considerable emphasis has been placed on the relation of LH to ovulation but this does not mean necessarily that it is the hormone chiefly responsible for rupture of the follicle.

In all instances, so far mentioned, in which ovulation has been evoked experimentally the possibility has existed that both follicle-stimulating and luteinizing actions have been present. This has been brought about either by endogenous gonadotropins or by the physiological nature of the gonadotropin injected. This has been true even in experiments in which, after follicular development, ovulation has been induced in hypophysectomized animals by the injection of pregnancy urine hormone. This chorionic gonadotropin is not entirely a luteinizing substance as is so often stated in the literature (see Engle, 1939, for references). Therefore, it seems possible that pre-ovulatory enlargement and ovulation in the normal estrous cycle may be accomplished by a balanced action of the two pituitary hormones, FSH and LH, not one, often referred to as "the ovulating hormone."

Analysis of the actions of FSH and LH in ovulation. Evidence in support of the theory that both FSH and LH are required for ovulation can be drawn from investigations in which partially fractionated pituitary preparations have been used under conditions that minimized or eliminated participation of the animal's own pituitary. Such studies include ovulation in anestrous and immature cats (Foster and Hisaw, 1935), juvenile rabbits (Foster and Fevold, 1938) and hypophysectomized adult rabbits (Foster, Foster and Hisaw, 1937). Follicular development in all instances was produced by daily injections of FSH for five days and ovulation was induced by a single intravenous injection of FSH, LH, or a mixture of the two hormones. In both cats and juvenile rabbits it was found that ovulation could be evoked by an adequate dose of either FSH or LH but a mixture of the two was more effective, as judged by the number of ruptured follicles. It was also found that when the amount of FSH in the ovulating mixture was kept constant and various amounts of LH were added, ovulation decreased as the concentration of LH increased until a point was reached at which no ovulation took place. When similar preparations were tested on hypophysectomized rabbits it was found that neither FSH nor LH alone could produce ovulation but a combination of the two was effective.

Greep, van Dyke and Chow (1942) tested the ovulating action of pure FSH and LH on normal estrous and post partum rabbits. Both hormones produced ovulation and LH was found slightly more effective than FSH. They also tested the effects of combined injections of the two hormones and demonstrated a potentiation but of a smaller degree than that obtained by Foster, Foster and Hisaw (1937). It is unfortunate that these pure preparations were not tested

also on hypophysectomized rabbits. However, these experiments, as a whole, tend to support the theory that ovulation results from the combined actions of FSH and LH.

Probably the most promising material for an analysis of the process of ovulation is the amphibian ovary. Since the first experiments on the induction of ovulation by Wolf (1929) on *Rana pipiens* and Houssay, Giusti, and Lascano-Gonzalez (1929) on *Bufo marinus* similar observations have been made by many investigators on various species of both Anura and Urodela. Only a few papers have been selected that have direct bearing on the present discussion and the reader is referred to a review by Creaser and Gorbman (1939) for a general consideration of the literature.

It has been found recently that ovulation can be produced in *Rana pipiens* by administering fractionated gonadotropins from mammalian pituitary glands (Wright and Hisaw, in press). This has made it possible to repeat on the frog several experiments that have been performed thus far only on mammals. One of these is the action of FSH and LH in the hypophysectomized frog. It was found that an FSH preparation would produce ovulation in the normal frog but could not do so in the hypophysectomized animal. However, both the normal and hypophysectomized animal would respond to combinations of FSH and LH.

Another effect produced by FSH, first observed in the frog, is a sensitization of the ovary to an ovulating stimulus. Rugh (1939) found that hypophysectomy greatly increased the sensitivity of the ovary to implanted pituitary tissue. Wright (1946) confirmed this observation and suggested that the effect was probably due to a release of gonadotropins from the pituitary during the operation. This idea is in agreement with the observations of Lane and Greep (1935) and Williams (1945) who found that pituitary ablation produced stimulation of the ovaries in rats. In the frog, one of the hormones released is apparently FSH. The injection of a small amount of an FSH preparation, that is insufficient for ovulation, will increase the responsiveness of the ovary in a normal frog. Also, hypophysectomized frogs given eight daily injections of an FSH preparation, adequate to ovulate a normal animal, did not ovulate, but their ovaries remained more responsive to an ovulating mixture of FSH and LH than those of a normal animal. The ovaries of similar animals, not treated for the same period, become refractory thus indicating that an FSH preparation also prevents involutionary changes in the follicles that ordinarily result from loss of the pituitary.

An advantage offered by the frog's ovary is that it will ovulate in vitro and the process can be watched. Heilbrunn, Daugherty, and Wilbur (1939) produced ovulation in *Rana pipiens* by suspending one ovary in Ringer's solution containing a triturated pituitary gland of a frog. Ryan and Grant (1940) obtained ovulation by putting small pieces of ovary in vials containing a suspension of frog pituitary tissue in Ringer's solution. Wright (1945) has made a rather extensive study of the conditions influencing ovulation of the frog's ovary in vitro. His experiments were conducted on bits of ovary including 15 to 30 ova which were suspended in vials containing 10 cc. of Holtfreter's fluid. This made it possible to have a large number of duplicates and controls from the

same ovary. Ovulation was induced by adding various amounts of a fine suspension of frog pituitary tissue. Probably the most important observation, as it relates to the present topic, is the influence of concentration of pituitary suspension on the percentage of ovulation. This was tested by adding to a series of vials containing tissue from the same ovary, different amounts of the suspension ranging from that equivalent to two pituitaries to $\frac{1}{512}$ of a gland. The results for such experiments show a gradual increase in the percentage of eggs ovulated until a concentration of $\frac{1}{8}$ to $\frac{1}{16}$ of a pituitary is reached after which there is a progressive decline. In fact, in some tests no ovulation took place at concentrations equivalent to one or two pituitary glands. The reason for the inefficiency of the higher concentrations is not known but the observations on juvenile rabbits by Foster and Fevold (1938) suggest that it might be correlated with the increase in amount of LH as more pituitary tissue was added to the solution.

A partial answer to this question probably can be found in the results for in vitro experiments in which mammalian gonadotropins were used. Wright and Hisaw (in press) have found it possible to induce ovulation in bits of frog ovaries in vitro with extracts of sheep pituitary glands under the same conditions as those just described. They found that an ovarian fragment in Holtfreter's fluid could not be made to ovulate by adding an FSH preparation. However, the ovarian tissue became much more responsive to mixtures of FSH and LH than bits of the same ovary that had not been treated with FSH. For example, ovulation of ovarian fragments of a normal ovary was not obtained with mixtures more dilute than $\frac{1}{4}$ unit of LH plus $\frac{1}{2}$ unit of FSH but similar bits of ovary treated with FSH responded to as little as $\frac{1}{16}$ unit of LH plus $\frac{1}{32}$ unit of FSH. The optimal balance between the two hormones for ovulating either normal or sensitized bits of ovary was not determined but it was found that $\frac{1}{2}$ unit of LH plus $\frac{1}{6}$ unit of FSH was ineffective while $\frac{1}{2}$ unit of LH plus 1 unit of FSH produced ovulation. Also, the effect of LH alone was not determined due to the lack of a preparation of sufficient purity.

These investigations on the frog are of general interest as they indicate that probably the physiological processes concerned with ovulation are basically the same in all vertebrates. It must be significant that FSH preparations do not cause ovulation in hypophysectomized rabbits, hypophysectomized frogs, nor in bits of frog ovaries in vitro, while proper combinations of FSH and LH will do so in all instances. Yet it is a fact that the information we now have is based for the most part on results in which pituitary preparations of questionable purity have been used. Such data are no doubt reliable in most respects but should be accepted with reservation until the effect of chemically pure pituitary gonadotropins have been investigated.

Conclusions on ovulation. The results for experiments on ovulation tend to support the theory that rupture of the follicle is brought about by the synergistic action of FSH and LH. Yet the quantitative relationship between these two hormones, that is most favorable for ovulation, has not been determined for a single species. It also seems likely that there is considerable variation and that

each species, though similar to all others, has its individual pecularities. This certainly seems true when one considers the responsiveness of some and the difficulties encountered with others. With regard to this one need only mention the discouraging experiences of those who have attempted to produce ovulation in primates (Hartman, 1938; Davis and Hellbaum, 1944; Abarbanel and Leathem, 1945).

It is also a problem as to how the proper balance between the pituitary hormones, at the time of ovulation, is achieved. It seems to be the general opinion that this is accomplished by an increase in secretion or release of hormones from the pituitary as a result of nervous stimulation at mating or in response to the ovarian hormones, estrogen and progesterone. A point made in the discussion is that estrogen is found in all vertebrates while progesterone probably is present only in mammals and therefore cannot be of general importance in ovulation. Although Everett has presented strong evidence in support of the possibility that small doses of progesterone stimulate the secretion of LH by the pituitary in rats, it is also true that larger doses and functional corpora lutea inhibit ovulation. The writer is inclined toward the opinion that these actions of progesterone are mammalian adaptations which assist in regulating the estrous cycle while among the vertebrates as a whole the action of estrogen on the pituitary is probably more important as far as ovulation is concerned. Also, one might expect that the pituitary would not respond to estrogen in those species that require the nervous stimulation of mating for ovulation. Bachman (1935) found this so for the rabbit, but whether it is generally true is not known. On the other hand, the pituitaries of those species that ovulate spontaneously should react to estrogen. Evidence for this has been presented for the rat and sheep but such information for other mammals is almost entirely lacking.

If it is agreed that the output of gonadotropins by the pituitary is increased during ovulation there is yet the question as to whether it concerns FSH, LH or both. An increased secretion of LH is mentioned most frequently in the literature. These statements concerning LH are probably based on the close association of luteinization with ovulation but at present there are no quantitative data that are suitable for settling this point. The results of research on the rabbit are the nearest approach. Hill (1934) found that mating in rabbits was followed by a rapid decrease in the ability of the pituitary to induce ovulation when tested in estrous does and Westman and Jacobsohn (1937) found a corresponding increase in the ovulating capacity of the blood. These observations demonstrate a greater output of gonadotropin but they are not quantitative in the sense that they indicate the amount of the hormone or hormones involved. The writer is of the opinion that the evidence is in favor of LH but these results could be explained by assuming an increase in LH, FSH or both as either or a combination of the two will cause ovulation in a normal estrous rabbit. There is much to commend in the idea expressed by Dempsey (1937) that it is not necessary to postulate wide fluctuations in FSH during the estrous cycle as all the phenomena could be explained on the assumption that FSH remains quite constant and ovulation results from an increase in LH. This and many similar

problems could be solved if accurate quantitative techniques were available for determining the FSH and LH content of unfractionated preparations of small samples of blood and single pituitary glands of laboratory animals.

It also is clear that before a complete explanation of ovulation can be given, the relative importance of FSH and LH must be determined. At present we have no reliable quantitative data on this point. It is true that important and suggestive observations of a quantitative nature have been made, but in all instances reasonable doubt can be raised either with regard to the purity of the pituitary preparations used, or the possibility of endogenous gonadotropins being present. In experiments on mammals these objections can be met by using pure FSH and LH and hypophysectomized animals. It also is possible that very valuable experimental material for investigations on ovulation can be found among the cold-blooded vertebrates. Amphibia hold the most promise and offer the advantage of studying ovulation in vitro. We now have the basic information, techniques, and materials for solving this problem and one might expect significant progress within the near future.

SUMMARY

Follicular development. The development of a Graafian follicle can be divided into four stages. (1) The first includes oogenesis, organization of the granulosa and theca interna, and growth up to the formation of the follicular antrum. In mammals the ovum attains approximately its full size. A characteristic of this period is that the growth processes probably are regulated by a self-contained system of organizers. (2) The second stage begins when the follicle gains competence to respond to the action of pituitary gonadotropins. It is marked by rapid growth, mitotic activity in the theca interna and granulosa, and multiplication of follicular blood vessels. (3) The third stage is introduced by a rapid falling off of mitotic rate in both theca interna and granulosa. The blood vessels continue to increase and reach full development in the fourth stage. This seems to be a period of differentiation, indicated by hypertrophy of the cells in the theca interna and granulosa. Increase in size of the follicle is due mostly to the accumulation of fluid in the antrum. (4) The fourth stage includes pre-ovulatory enlargement of the follicle and ovulation. The chief features are the rapid secretion of follicular liquor, marked hyperemia of the follicle, and usually the ovum undergoes the first polar division.

The physiological processes of the last three stages proceed under the coordinated actions of the pituitary and the ovarian hormones.

Ovulation. Much regarding the physiology of ovulation remains obscure but the results of recent research warrant the following tentative conclusions: (1) That pre-ovulatory enlargement and rupture of the follicle is produced by the joint action of FSH and LH. (2) That neither FSH nor LH acting alone can cause ovulation. (3) Ovulation is initiated by an increase in the secretion of gonadotropins by the pituitary and probably this mostly concerns LH. (4) This increased output of gonadotropins is caused, in species that ovulate spontaneously, by the action of estrogen and possibly progesterone on the pituitary, and

probably by nervous stimulation of the pituitary in those species that require mating for ovulation. (5) Another possibility worth considering is that in certain species the sensitizing effect of FSH on the follicle (frog) may assist the ovulatory reaction or even make it possible for the follicle to respond to the existing concentration of gonadotropins thus making an increase unnecessary for ovulation.

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THE RENAL ORIGIN OF HYPERTENSION

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There is no reasonable doubt that some cases of human hypertension are of renal origin. The unsolved problem is whether many or most of those still referred to as "essential" are also of renal origin. The purpose of this review is to supplement the one made in 1940, in this Journal, by Blalock (24) and to summarize and evaluate the pertinent evidence that has now accumulated for or against the renal origin of human essential hypertension. Only relatively recent original references will be given here; for many of the established facts reference will be made to the bibliographies of previous reviews.

In the past, the renal origin of the elevated blood pressure in human essential hypertension has been excluded, by definition. Essential hypertension has usually been defined as persistent elevation of systolic and diastolic pressures, without accompanying renal disease, as evidenced by impairment of renal excretory function, when studied by the usual methods. Dalton and Nuzum (74) believe that critical statistical analysis of data on renal function in cases of human essential hypertension does show, in most cases, some reduction in the concentration of the urine and excretion of phenolsulphonephthalein. Yet it is a fact, known for many years, that in patients with long-standing hypertension, without obvious manifestations of renal excretory impairment during life, intrarenal arterial and arteriolar sclerosis, or even true nephrosclerosis, frequently may be found at autopsy (16, 109, 219, 254, 273). The unanswered questions are: (1) whether the hypertension or the vascular disease comes first, and (2) whether there is a causative relationship between the two conditions. Even to-day, as was the case 50 years ago (3, 214), some authors (153, 235, 306, 384) still regard the hypertension as primary, and of unknown origin, and the arterial and arteriolar sclerosis, both intrarenal and systemic, as independent of (3) or secondary to (214) the prolonged mechanical stretching of the walls of the blood vessels. Probably the only reason why some of these authors (153) concede that the hypertension of glomerulonephritis, pyelonephritis, and polycystic disease of the kidneys is induced by the renal disease is because it is known that these diseases of the kidney precede the hypertension. It is therefore not reasonable to contend that the hypertension is the cause of these inflammatory or developmental renal lesions. It is possible, however, to contend that the high blood pressure, or rather the increased bursting tension of the blood vessels, may be a mechanical cause of both intrarenal as well as systemic arteriosclerosis; therefore, this view has many modern adherents (153, 235, 274, 306, 384). These authors do not concede that the hypertension is a consequence of the renal vascular disease, although they agree that the renal disease may play a secondary part in maintaining the hypertension which is initiated either by a nervous (306) or a humoral (153) non-renal mechanism. Smith, Goldring and Chasis (384) regard the kidney as "the victim, rather than culprit" and

Schroeder (363) believes that essential hypertension may be the result of the combination of a number of factors.

If there is any one thing that is definitely established about the mechanism of the elevation of blood pressure, it is that widespread increase of peripheral (including splanchnic) vascular resistance is the determining factor (387) and that it is the tone of the systemic arterioles that is the determinant of peripheral vascular resistance. The old idea of v. Basch (3) that the arteriolosclerosis can be so generalized as to be a mechanical, organic cause of increased peripheral vascular resistance still persists, but no one has ever demonstrated such widespread disease of the blood vessels. One of the striking things about arteriosclerosis in man is how restricted the vascular disease is to certain organs and parts of the body, and especially how common it is in the kidneys (16, 109, 219, 254, 273). In this connection, the statement recently made (153) that "the kidneys are rarely involved to a significant degree in hypertensive disease; only about 8 per cent of all patients die of uremia", is misleading, for it attaches too much importance to renal excretion and ignores the important fact that the kidneys may be the seat of advanced vascular disease without significant disturbance of renal excretory function detectable by the most recent methods (108, 112). Finally, there is the view of the author (144) that the arterial and arteriolar sclerosis are primary, but of unknown origin, and that when the vascular disease affects the kidneys to a degree sufficient to produce adequate disturbance of intrarenal hemodynamics, a mechanism is initiated which brings about the increased peripheral vascular resistance that determines the hypertension. This view implies, of course, that any other pathologic process in the kidney which is capable of inducing the same disturbance of intrarenal hemodynamics would also be capable of initiating the mechanism that results in hypertension (76, 110, 202, 203, 206, 428).

It is obviously impossible to solve a problem of this kind by the study of patients, clinically, or by the examination of specimens of tissues obtained at autopsy. The arteriolar sclerosis of the kidneys, which has been reported by many authors (16, 109, 219, 254, 273), as an almost invariable finding in cases of human essential hypertension, at autopsy, is interpreted by some as proof for the view that the hypertension comes first and produces the renal vascular disease (44, 397). The possible causative relationship between the two conditions could conceivably be investigated by the examination of biopsy specimens of kidney obtained from the same individuals, at intervals, before and after the development of the hypertension. Since there is no way of telling with certainty which persons will develop hypertension, such a study of human beings is not feasible. An approach to this problem has been made by the examination of biopsy specimens of kidneys in cases of established human hypertension, after the condition had been in existence for various lengths of time. This study by Castleman and Smithwick (44, 397) has not led to a solution of the problem, for obvious reasons; but it is well to examine what it has contributed.

In a series of patients with hypertension that had existed for variable periods, biopsy specimens of the kidneys were examined for vascular changes. In these

small specimens in which, at best, only a few cross sections of independent arterioles could be observed, the changes were classified arbitrarily from 0 to ++++ on the basis of the amount of hyaline thickening of the wall and stenosis of the lumen. Despite the views and conclusions of the authors, it is an interesting and indeed an extraordinary fact that some vascular disease was found in 92 per cent of the specimens and that in about 50 per cent it was from moderate to severe. From the study of such a small specimen it is obviously impossible to tell whether the vascular disease is more or less pronounced in the remainder of the kidney. It is hazardous, therefore, to extrapolate from the evaluation of such a minute specimen of heterogeneous tissue to an estimate of the average condition of a single constituent, the arterioles, of the entire organ. An important fact, frequently overlooked, is that the larger intrarenal arteries are rarely included in such biopsy specimens, because the specimens are taken from the peripheral portion of the cortex. Even if the appearance of a few arterioles can give some estimate of the average change in similar vessels of the remainder of the kidney, yet it can give no idea of the state of the larger intrarenal vessels, or of the hemodynamic state of the entire kidney. It should be obvious that stenosis of one large intrarenal artery could account for great hemodynamic disturbance in a large mass of kidney, supplied by many arterioles that are not themselves diseased. Arteriosclerosis, with stenosis, of the larger *intrarenal* vessels is a common accompaniment of renal arteriolosclerosis. The possible contribution of stenosing sclerosis of the larger intrarenal arteries, and even of the main extrarenal artery, to the disturbance of intrarenal hemodynamics has been underestimated. Blackman (23) has shown how common it is to find at autopsy some degree of stenosis even of the main renal artery in hypertensive patients. At no time has it been asserted that stenosis of the main renal artery, unilateral or bilateral, is a common cause of, or frequent finding in, cases of human essential hypertension. This is why it is difficult to understand why Oppenheimer, Klemperer and Moschkowitz (287), and Lisa, Eckstein and Solomon (254), should make a special point of emphasizing that in many of their cases of hypertension in which pronounced stenosis of one or both main renal arteries was found, there was also significant intrarenal arteriolosclerosis. This is admitted freely by the author.

Experimental Renal Hypertension. The working hypothesis of the initial attempt (152) to produce experimental hypertension that might simulate human benign essential hypertension unaccompanied by disturbance of renal excretory function was based upon the assumption that, if the hypertension be the result of the *intrarenal* arterial and arteriolosclerosis, then the real cause of the elevated blood pressure might be the functional disturbance of renal hemodynamics produced by the stenosing vascular disease in the kidneys. It was considered that, if this were true, then the experimental production of such a disturbance of intrarenal hemodynamics by any means, even by constriction of the main renal arteries, by means of a clamp, might be followed by the development of hypertension without accompanying disturbance of renal excretory function. The clamp for the constriction of the main renal artery was not devised in order

to reproduce the effect of arteriosclerosis, with stenosis, of the main renal artery alone. It was fully recognized that this condition is not commonly found in patients with essential hypertension, although it has been shown to occur not infrequently (23). The clamp was developed as a device with which to reproduce experimentally, in animals, the probable hemodynamic disturbance in the kidney produced by *intrarenal* arterial and arteriolar sclerosis, so frequently found in persons with essential hypertension, or any other of a variety of pathologic processes, including stenosis of the main renal artery, which could produce a similar intrarenal circulatory disturbance. On the basis of an anatomical study of kidneys in human hypertensive persons Yuile (446) later found that intrinsic or extrinsic obstruction of the main renal artery simulating the renal clamp does occur, and concluded "that no evidence opposed to the view that experimentally produced hypertension has a human counterpart was encountered".

That hypertension which is unquestionably of renal origin, and which simulates human hypertension associated with renal vascular disease, has been produced experimentally, by constriction of the main renal artery, is now beyond question, for this has been fully confirmed by many investigators (92, 99, 102, 161, 163, 178, 195, 230, 293, 352), some of whom have employed modifications or different devices for the purpose of producing similar intrarenal hemodynamic disturbance.

In this review the account of the production of experimental renal hypertension will be made brief, because there have been many previous publications (146, 148, 149) about this phase of the subject. The original production (152) of experimental renal hypertension was accomplished by permanent constriction (not occlusion) of the main renal artery by means of a special silver clamp devised for the purpose. The effect of the clamp was to restrict the inflow of blood to the kidney, with resultant disturbance of intrarenal hemodynamics, the exact nature of which has not yet been definitely established.

There is no doubt that the immediate effect of constriction of the main renal artery is to restrict the flow of blood through the kidney (152, 251). Whether this effect persists indefinitely has not yet been determined with certainty (368). Restriction of the outflow of blood from the kidney by partial constriction of the main renal vein is also followed by transient hypertension (17, 118). It has been shown by Mann and collaborators (262) that it is necessary to reduce the caliber of an artery considerably before an appreciable effect on blood flow through the vessels is produced. All attempts to produce widespread intrarenal circulatory disturbance by emboli of various types had failed to produce hypertension (9, 42, 376). Reproduction of the widespread intrarenal arteriolar sclerosis would have been desirable, but it had not yet been accomplished at that time and it has not yet been produced in the dog. It is only recently that the production of so-called nephrosclerosis in chicks and rats has been reported by Selye and collaborators (371, 372, 373, 374, 375). There is no convincing proof in any of these studies that widespread intrarenal arteriolar sclerosis has been produced. The changes described are mainly parenchymatous degeneration and interstitial fibrosis, with an occasional arteriole showing fibrosis,

hyalinization and even necrosis. Some of these animals developed elevated blood pressure (372). The production of nephrosclerosis even in the chick and rat has not yet been confirmed; but others (38) have reported the hypertensive effect on the rat of repeated injections of desoxycorticosterone acetate.

Whether the results obtained by means of constriction of the main renal arteries can be applied completely to the problem of human essential hypertension still remains unanswered; but there is adequate indication that experimental renal hypertension and human essential hypertension are closely similar, if not identical, and that the results obtained in studies of pathogenesis, prevention, or treatment of the one may be applied directly to the other.

There are, however, those (153, 235, 254, 287) who refuse to assign great significance to these experiments, because, in human hypertension, the main renal artery is not commonly stenotic and therefore the vascular disease of the human kidney is not identical with experimental constriction of the main renal artery. What is forgotten, or ignored, by these authors is that constriction of the main renal artery was an expedient resorted to experimentally because it was the only available method whereby to produce a disturbance of the intrarenal circulation that might simulate the most probable effect of intrarenal stenosing arterial and arteriolar sclerosis. To think that the experimental type of hypertension is not exactly like human benign or malignant essential hypertension, because the main renal artery in human beings with hypertension is rarely stenotic (254), is to misunderstand the whole problem and the main purpose of the procedure which was used for the production of experimental renal hypertension. Such a concession as that made by Goldring and Chasis (153), namely, that a mechanism analogous to that of experimental renal hypertension can initiate hypertension in man but that this occurs but rarely, is gratuitous, and again ignores the probable similarity between the effect of widespread *intrarenal* vascular disease and experimentally produced stenosis of a main renal artery, which was the basis of the original experimental investigation (152). Recognition of this similarity is essential to a proper understanding and evaluation of the contribution made by the production of experimental hypertension by constriction of the main renal artery.

Temporary Hypertension Due to Constriction of One Main Renal Artery. Temporary hypertension which gradually returns to normal, in about four to six weeks, has been produced by constriction of one main renal artery. If the clamp is removed, and the circulation of the kidney restored to normal, or if the one kidney with the renal artery constricted is excised, the blood pressure returns to normal in 24 hours or less (25, 146, 223). In some animals, especially rats, goats, sheep, and even an occasional dog, unilateral constriction of the main renal artery is followed by the development of hypertension which persists for many months. Even in such animals the blood pressure returns to normal in 24 hours, or less, if the kidney with constricted renal artery is excised. It has been shown that, in dogs at least, the blood pressure may return to normal in as little as six hours.

In addition to affording evidence in favor of the renal origin of this type of

experimental hypertension, the significance of these observations on animals for the problem of hypertension in man has been the recognition of the existence of persistent hypertension associated with unilateral renal disease in man and of a possible cure of the hypertension, by excision of the diseased kidney, provided the other kidney is normal. The subject of the existence of hypertension associated with unilateral disease in man, and the return of the blood pressure to normal, in some patients, as the result of the removal of the diseased kidney, has already been reviewed (1) and more cases, some with return of blood pressure to normal after unilateral nephrectomy, have now been reported (10, 11, 12, 18, 27, 29, 30, 124, 141, 202, 255, 270, 316, 317, 341, 344, 346, 354, 376, 377, 379, 381, 384, 392, 394, 399, 427, 428, 429, 444, 445).

There is no convincing proof that intermittent total renal ischemia results in permanent elevation of blood pressure (148). There is every reason to believe that those who still insist that this does occur, in an exceptional case, were really dealing with permanent disturbance of renal circulation in an occasional animal (5, 257).

Persistent Hypertension Due to Constriction of Both Main Renal Arteries, and other Measures. Persistent hypertension has now been produced in dogs, monkeys, rabbits, sheep and goats (146), but not in the frog (408), by the constriction of both main renal arteries (146). As in the case of unilateral constriction of the main renal artery, a significant rise of blood pressure does not usually manifest itself in less than 24 hours, and often longer, although there is some evidence that the hypertensive effect may at least be initiated soon after the constriction (405). Hypertension due to constriction of one main renal artery may be made permanent by subsequent excision of the contralateral normal kidney (144, 225), by constriction of the main renal artery of the contralateral kidney (152), by occlusion of the ureter of the contralateral kidney (147, 271), or by the enclosure of one or both kidneys (preferably decapsulated) in a fishskin condom (146, 149). This type of membrane does not induce the development of a thick hull of connective tissue around the kidney, such as is induced by cellophane, collodion or silk (294, 296), but it does prevent the development of accessory circulation to the kidney. If the constriction of both main renal arteries is made moderate, the hypertension which results is not accompanied by detectable disturbance of renal excretory function—the benign phase (60, 152). If the constriction of both main renal arteries is made great, at the first operation, there is likely to be definite disturbance of renal excretory function, and fatal uremia may develop—the malignant phase (145, 147, 443). That complete renal ischemia for a few hours may result in anatomical and functional damage has been shown by Selkurt (370).

In those animals in which the blood pressure returns to normal after unilateral or bilateral constriction of the main renal arteries, the examination of the kidneys frequently shows that the naturally abundant, but poorly developed, accessory circulation to the kidney has become strikingly prominent, with large arterial vessels entering the cortex of the kidney from various surrounding sources. This observation indicates the possibility of treating experimental renal hy-

pertension by inducing the development of the accessory circulation to the kidney. Although the hypotensive effect of this procedure has been demonstrated experimentally, in dogs, by decapsulation and nephro-omentopexy (45) and by implantation of a part of the kidney into the spleen (spleno-renopexy) (425), yet this has not proved a fruitful lead for the treatment of human hypertension.

Pathologic Changes in the Organs of Animals with Persistent Hypertension. In the benign phase, in animals, even after six years of persistent hypertension, no significant pathologic changes develop in the aorta or in the large or small arteries. The only changes observed in the vascular system of these animals are slight to moderate hypertrophy of the heart (53) and thickening of the media of the large and small arteries (144). The results of these experiments, therefore, afford no proof for the view that hypertension *per se* is a sufficient condition for the production of generalized arterial or arteriolar sclerosis in the animals used. The adenomatous hyperplasia of the adrenal cortex reported in cases of essential human hypertension (345) has not been confirmed by others and has not been reported in animals with experimental renal hypertension.

In the malignant phase in animals, even when it terminates fatally in as little as 48 to 72 hours, the most profound changes occur in the blood vessels of the dog (147) or rabbit (322, 439) similar to those of the terminal phase of human malignant hypertension. In the aorta there is only interstitial edema, mainly of the media, but in the small arteries and arterioles, necrosis and fibrinoid degeneration occur, with or without acute inflammation in and around the wall of the vessels. The arteriolonecrosis and necrotizing arteriolitis, observed in many organs, are indistinguishable from similar changes observed in the terminal phase of malignant human hypertension.

The vascular changes of the malignant phase do not occur in the blood vessels of bilaterally nephrectomized animals that develop profound azotemia, without elevation of blood pressure, or in animals with great degree of hypertension of long standing, without renal excretory functional disturbance. They do not occur, nor does hypertension develop, in dogs in which the urine is deviated into the blood (139). In the malignant phase, in animals, the changes do not occur in the vessels of the kidney beyond the site of the clamp, where there is no hypertension, and where hypotension probably exists. For these reasons, it has been considered that a combination of impairment of renal function and increased bursting tension of the vascular wall are necessary conditions for the production of the pathologic changes in the vessels and that, even in human beings, these changes usually occur only terminally. The idea that these changes are induced by long-standing (months to years) intense vasospasm (409) is not upheld by these experiments. The inflammation around the blood vessels is probably only a reaction to the degeneration and necrosis of the walls of the arterioles, but, of course, may be caused by the same agent that produces the necrosis. These pathologic changes in the arterioles are not to be confused with arteriolosclerosis and are certainly not to be used as proof that hypertension itself can produce true arterio- or arteriolosclerosis. This erroneous interpretation has been made (90, 274).

We have not been able to confirm the observation (242, 243, 442) of vascular lesions of the malignant phase in animals that have been injected even with large quantities of renin, subcutaneously, intramuscularly or intravenously. This may be due to the difference in the method of preparation and purity of the renin employed.

Some investigators have produced lesions of arterioles which they consider similar to those of the malignant phase, by repeated intravenous injections of tyramine (93), but there is no good reason for believing that tyramine is responsible for the lesions in malignant hypertension.

The changes in the kidneys themselves depend upon the degree of constriction of the main renal artery and may vary from no detectable change, except in the mitochondria of the proximal convoluted tubules (101), to partial or even complete necrosis, or atrophy and fibrosis of the kidney (152). The conclusion of Wilson and Byrom (436, 437, 438) confirmed by others (122) that hypertension *per se* can produce the characteristic malignant arteriolar lesions in the rat, with hypertension due to constriction of one main renal artery, has not been substantiated in experimental renal hypertension in the dog, or any animal other than the rat (144, 147), and is probably based on the erroneous assumption that the contralateral kidney was normal. All the glomerular and interstitial inflammatory lesions described by these authors in the contralateral, supposedly normal kidney, have been observed by the author in rats with normal blood pressure. The author's explanation of the changes in the arterioles of the contralateral kidney, observed by Wilson and Byrom, and the others, is that they were unaware of the frequent existence of hydronephrosis or pyelonephritis in one or both kidneys of adult rats, and that, by constriction of one main renal artery, they were dealing, in some of these rats, with bilateral renal disease.

Pathogenesis of Experimental Renal Hypertension. This has been the subject of many publications, since the production of experimental renal hypertension was announced (152). Most of these accounts have dealt with the possible application of the observations on animals to the problem of the pathogenesis of human essential hypertension (24, 25, 146, 148, 208, 224, 235, 236, 271, 279, 280, 303, 306, 378, 384).

Progress in the acquisition of knowledge about the pathogenesis of human hypertension has been delayed because, in human beings, it is rarely if ever possible to study the patient before and after the development of the elevated blood pressure. The production of the counterpart of the benign and malignant phases of human hypertension has made possible the investigation of the probable pathogenesis of the high blood pressure, even in man. As soon as this was accomplished, the possible mechanisms involved in the elevation of the blood pressure became obvious: 1, afferent stimuli from the nerve endings in the kidneys to the vasomotor center or sympathetic ganglia, with resultant generalized vasoconstriction and consequent elevation of blood pressure; 2, afferent stimuli from the kidneys, with resultant output of an increased amount of some internal secretion which produces vasoconstriction either by central or peripheral action; 3, the accumulation or new formation of some substance, probably of renal origin, or a disturbance of equilibrium between substances already present

in the blood, resulting in a pressor effect like that of some of the known pressor substances.

Neurogenic Mechanism. In rapid succession the possible part played by various portions of the nervous system was investigated. It was soon shown that experimental renal hypertension is not caused by a nervous reflex from the kidney, affecting the vasomotor mechanism of the body. Renal denervation (53, 293); bilateral supradiaphragmatic excision of splanchnic nerves and lower dorsal sympathetic ganglia (148); subdiaphragmatic splanchnicectomy, with excision of the celiac and upper lumbar ganglia (25); bilateral section of the anterior nerve roots, from the sixth dorsal to the second lumbar inclusive (148); pithing (destruction of the entire spinal cord) (49, 143) and complete sympathectomy, including denervation of the heart (7, 117, 199) neither prevent, nor abolish the hypertension which results from the constriction of the main renal artery. Under pentothal anesthesia, experimental renal hypertension persists, whereas known neurogenic hypertension disappears (305). These results eliminated a nervous reflex originating in the kidney as the cause of the hypertension and indicated that a humoral mechanism was probably the cause. Ogden (283) however, who concedes that the renal humoral pressor mechanism initiates experimental renal hypertension, believes that he has demonstrated, by experiments on rats, that it is superseded later by a neurogenic mechanism mediated through the sympathetic nervous system. More work on this phase of the subject is required.

Although there are those who still cling to the idea that essential hypertension is endocrinogenic, and due to some disturbance of the hypophysis, yet the experiments on animals fail to confirm this. Hypophysectomy (8, 103, 148, 287, 315) has no definite effect. There is no definite proof (114) that the activity of the posterior lobe of the pituitary is altered in experimental renal hypertension. There is no difference between hypertensive and normal dogs insofar as the quantity of antidiuretic principle in the urine is concerned. During dehydration it is increased in both. Removal of the posterior pituitary does not interfere with the development of, or lower, experimental renal hypertension in the rat (286). The same holds true for the removal of the entire pituitary in dogs (148, 315). Thyroidectomy, gonadectomy and pancreatectomy do not affect experimental renal hypertension (148).

Of the endocrine organs, the only one that may possibly play a significant, even if only a secondary part, is the adrenal (79), although this conclusion is contested (57, 351) on the basis of inadequate evidence. There is no evidence to indicate that the medulla of the adrenals plays a part (152), but there is definite indication that the adrenal cortex may play a secondary part (148, 431) in experimental renal hypertension, although there are those (350, 351) who do not concede this. Excision of both adrenals in dogs interferes with the development of hypertension due to constriction of the main renal arteries, unless adequate supportive and substitution therapy are given (148). The part possibly played by the adrenal cortex will be discussed further under the heading of the humoral mechanism.

Recently Victor (406) reported the production of hypertension in dogs by ligation of "hilar artery and vein" and of "grossly visible arteries and veins at either the superior or inferior pole" of one adrenal. Although he does not state this, yet it is evident that nerve fibers must have been included in these ligatures. Victor does not explain or speculate about the mechanism of the hypertension. We have tied off the hilar part of the adrenal vein and the vessels going to and from the upper or the lower pole of one adrenal, in seven dogs, without noting any hypertensive effect. This contribution by Victor must await confirmation and elucidation.

The Humoral Mechanism of Experimental Renal Hypertension. The basic observation that constriction of the blood supply to the kidney produces experimental renal hypertension, and the elimination of a possible primary part played by the nervous and endocrine systems, stimulated the search for the humoral mechanism of renal origin most probably responsible for the elevation of blood pressure in this type of hypertension, and possibly also in human essential hypertension. Most recent contributions to the subject have dealt with the nature of this mechanism, about which two distinct viewpoints have been adopted and investigated: 1, that a kidney the seat of certain pathologic conditions may be the source of a substance which, when it enters the circulation, raises the blood pressure (297); 2, that the normal kidney may be the source of a substance the absence, destruction or neutralization of which results in hypertension (172). Most of the evidence, both old and recent, has been in favor of the existence of the first mechanism (252, 378), although there are still some who are opposed to this theory (396).

It has been shown that interference with the blood supply to any other organ but the kidney does not result in either temporary (253) or permanent (152) elevation of blood pressure. Constriction of the celiac axis and superior mesenteric artery (256), of the femoral and splenic arteries (152) and of the aorta below the origin of both main renal arteries (148) does not produce a rise of blood pressure; but constriction of the aorta immediately above the site of origin of the main renal arteries is followed, in about 24 hours, by the elevation of the blood pressure (146, 353, 388). Steele and Cohn (389) believe that the hypertension observed in human cases of coarctation of the aorta is like the effect of constricting the aorta above the origin of both renal arteries. Anastomosis between renal artery and renal vein does not result in hypertension (238). The removal of both kidneys, although profound azotemia is a consequence, is not followed by the development of hypertension; therefore, renal excretory insufficiency by itself, is not a sufficient condition for its development. This is also well demonstrated by the fact that in acute nephrosis, with uremia, produced by various metallic poisonings, in animals and man, the blood pressure rarely becomes significantly elevated.

The first, but indirect, evidence in favor of the probable existence of a renal humoral mechanism for the elevation of blood pressure in experimental renal hypertension was produced by the occlusion of both main renal veins at the same time that both main renal arteries were constricted sufficiently to

produce hypertension. The blood pressure did not become elevated, although the dogs died in profound uremia (148). The arteriolar lesions of the malignant phase did not develop in these dogs. Shunting of the venous blood of a dog from the only kidney, with renal artery constricted, through the liver, by means of a reversed Eck fistula, had no effect in either preventing the development of hypertension or lowering the blood pressure (50, 405). Any important effect of the liver on the pressor substance of renal origin was thus excluded.

A more direct demonstration of a possible humoral mechanism occurred when it was found that if a kidney was transplanted to the neck (24, 26, 105, 211) or groin (142, 336) of a bilaterally nephrectomized dog or rabbit the usual pressor effect resulted when the renal artery of the transplanted kidney, with no nervous connection with the rest of the body, was constricted. Also, the transplantation of a partially or completely ischemic kidney from one dog to the neck of a bilaterally nephrectomized dog, resulted in a temporary elevation of the blood pressure, after the circulation of the ischemic kidney was restored (33).

Corrigan and Pines (64) reported a fall of blood pressure in dogs with experimental renal hypertension, as a result of renopexy (elevation of the kidney for a distance of about 5 cm.) which was supposed to increase the outflow of blood from the renal vein. Why the blood pressure should fall as a result of this procedure is not clear. This experiment requires confirmation before it can be accepted and evaluated. It may have some significance in connection with the problem of hypertension associated with ptosis of the kidney (266). The study of Gabriele (137), however, indicates that such a change of position of the kidney is likely to reduce profoundly, rather than increase, outflow of blood from the renal vein, because the piezometric angle of the renal artery is made relatively acute by this change of position.

It has been shown that when the main renal artery (54, 55, 398), or the entire renal pedicle (120, 136, 248, 253) is occluded, for varying periods, in the cat, rat and dog, a prompt rise of blood pressure follows the release of the clamp and restoration of the circulation through the kidney, and this occurs even in animals with kidney denervated and central nervous system ablated (54). This effect is not prevented by 933F[1] (248) or by sodium cyanide (263); but previous establishment of tachyphylaxis in cats interferes with the phenomenon (248) and it does not occur when similar ischemia of other organs is produced (253). This study was carried further by the demonstration of Prinzmetal, Lewis and Leo (335) and others (233) that saline perfusate of such a totally ischemic kidney produced a definite rise of blood pressure when it was injected intravenously into other animals of the same or different species. Boylston and collaborators (28) did not find a pressor substance in the saline perfusate of a partially ischemic kidney, but this may be explained by the prolonged perfusion which they practiced.

The presence of a vasoconstrictor substance in the blood plasma from the renal vein of a dog with experimental renal hypertension due to incomplete constriction of the main renal artery has been demonstrated by some (32, 105, 197,

[1] This is piperidomethyl-benzodioxane, which is usually referred to as Fournier 933 or 933F.

212, 213 398) and not by others (265), by the Läwen-Trendelenburg technique, in the South American toad. Some investigators (126, 198, 223, 334) failed to demonstrate pressor substance in the systemic blood of hypertensive dogs or human beings and there was also no effect of blood from hypertensive dogs on the tonus of surviving arterial rings (422, 423). But Solandt and collaborators (385) did observe a definite rise in the blood pressure of a bilaterally nephrectomized dog to which was given a direct transfusion from a hypertensive dog; and Braun Menendez and Fasciolo (32) obtained a pressor effect in a normal dog as a result of the intravenous injection of 100 cc. of renal venous blood from the transplanted renal ischemic kidney of another dog.

That the hypothetical effective vasoconstrictor substance is not sympathicomimetic is shown by the fact that 933F (piperidomethyl-benzodioxane) does not have any greater effect on the blood pressure of a hypertensive animal than of a normal one, whereas, in both, the effect of epinephrine is completely reversed by an injection of 933F (119).

All the work outlined above pointed directly to a chemical agent of renal origin as the probable cause of the hypertension. The search for this agent began with the resurrection of an old observation made by Tigerstedt and Bergman (402) confirmed by others (187, 188, 197), namely, the presence of a substance in the crude saline extract of a normal rabbit kidney, capable of inducing a pressor effect when injected intravenously into a normal rabbit. This substance, which Tigerstedt and Bergman called Re-nin′ (re′-nin, in English) is now accepted as the basic substance of the humoral mechanism of experimental renal hypertension. Prinzmetal and collaborators (333) and others (433) found a greater amount of this pressor substance in the kidneys of dogs, but not rabbits, with experimental renal hypertension, and of hypertensive persons (393). Although this substance seemed to possess at least some of the basic requirements for the hypothetical pressor substance of experimental renal hypertension, yet its lack of vasoconstrictor properties (121) led two independent groups of investigators (36, 233, 234, 277, 278, 287, 308) to the realization that renin is not a direct pressor substance, but a key substance (241, 245) of the humoral mechanism which acts upon a substrate in the blood, to form a new substance, that is vasoconstrictor and therefore pressor.

In 1940, Landis (236) wrote, "The evidence that renal ischemia raises the blood pressure by a humoral mechanism seems unassailable". Yet Dock (88, 89), as the result of some acute experiments, has arrived at the conclusion that there is no peripheral vasoconstrictor in the blood of the hypertensive rabbit. Because the pithing of a normal rabbit, with blood pressure kept elevated by an infusion of renin, did not result in the fall of blood pressure (unless the infusion was stopped) while it did fall when a hypertensive rabbit (not receiving renin) was pithed, he concluded that in the latter the normal vasomotor mechanism was called into play and the vasoconstriction was abolished. A possible explanation, which has not been tested, is that the immediate effect of pithing is, temporarily at least, to abolish the output of the primary constituent of the humoral mechanism. This should be investigated.

In recent years, as a result of work on shock, evidence has been accumulating

which indicates that the normal kidney may play a part, even in the homeostatic regulation of normal blood pressure, through the humoral mechanism of renal origin which begins with the secretion of renin (184, 207, 215, 284, 355). There are those who doubt this. The low blood pressure in shock (hemorrhagic or otherwise) induces the secretion of renin and the formation of hypertensin (56, 83) in the blood. Shock due to hemorrhage produces a lowering of hypertensinogen (56, 83) in the blood of otherwise normal dogs but not in nephrectomized dogs or in dogs treated with KCN.

A generally accepted terminology for the constituents of the humoral mechanism has not yet been established, and none of the substances has been isolated in pure form. At the present time, the mechanism is supposed to be as follows, and the following names are in use by different investigators for the various substances which constitute this mechanism:

Renin
an enzyme from the kidney, enters the bloodstream through the renal vein, and acts upon

Hypertensinogen
Prehypertensin, Hypertensin-precusor Renin-substrate
a globulin in the blood plasma, to form

Hypertensin
Angiotonin
a polypeptide, which is the active vasoconstrictor substance, and which can be inactivated by

Hypertensinase
an enzyme in the blood and in extracts of some organs.

The name renin activator, originally proposed for the stubstrate, by Page, should certainly be abandoned, because the term is misleading, since the substance it represents does not activate anything, and is actually acted upon by the renin. It has been shown that it is contained in the α_2 globulin fraction (327).

Agreement upon a single nomenclature would be highly desirable, and the author suggests the acceptance of renin, hypertensinogen, hypertensin and hypertensinase as the most appropriate names for the constituents of the humoral mechanism of renal hypertension.

The following is a brief summary of the chemical and physiological properties of these substances:

Renin. Chemical properties. Renin has not yet been isolated in pure form, although several attempts to purify it have been made (22, 52, 84, 104, 191, 228, 322, 358). All that can be said is that it is either a protein, a protein-like substance, or contains protein as a contaminant (181, 191, 252, 321). It is present in the press-juice (187, 188) or in various extracts (35, 196, 218, 241, 321, 325, 329, 396, 441) of renal cortex (not medulla) and does not occur in similar extracts of other organs (spleen, liver) (121). It is thermolabile (destroyed by heat above 56°C) and not dialyzable. In an electric field, renin migrates to the cathode, the isoelectric point being between pH 6.5 and 7.5. The results of studies on the behavior of renin subjected to precipitation with ammonium

sulphate and dialysis suggest that it is a pseudoglobulin (181) but this has not been established beyond question. Most investigators regard it as a proteolytic enzyme (278, 309) extractable by various methods from all mammalian normal kidneys and from the kidneys of other animals (321, 434, 440). Plentl and Page (328) found in their purified renin the enzymes pepsin, trypsin, cathepsin, aminopeptidase and carboxypeptidase. They were not able to identify one of these enzymes as being specifically the enzyme renin, although they were able to exclude aminopeptidase and carboxypeptidase. But Winternitz and collaborators (441) have asserted that renin, in very pure form, is free of ordinary enzymatic activity. Various methods for extraction and purification have been described (52, 181, 252).

The preparation and isolation of renin, by extraction, are best carried out under aerobic conditions, because anaerobic conditions favor the formation of pressor amines, tyramine, isoamylamine and probably ethylamine (91). Kidneys from young rats have been reported to contain more renin than those from old rats; but old rats are more sensitive to parenterally administered renin (182). This study should be repeated on rats and other animals by use of more recently developed methods for extraction and assay.

Physiological properties. Renin is effective in raising blood pressure, when it is injected intravenously into animals, but it is not vasoconstrictor when dissolved in saline and perfused through a circulatory system from which the blood has been washed out. It is not a direct pressor substance (36). The physiological effects of renin injected intravenously into a normal animal are identical with the hemodynamic state of animals with experimental renal hypertension. It is not species specific for animals; all renin, including human, when injected intravenously, produces a pressor effect in all animals. But no animal renin so far produced has a pressor effect in man, who responds only to human renin (403). The pressor effect of renin is produced indirectly by the interaction of renin and hypertensinogen, a globulin in the blood plasma. This results in the formation of a new substance, hypertensin, which is the effective vasoconstrictor and therefore the true pressor substance. The site of action of renin itself is therefore in the blood and not on the vasomotor endings of the peripheral arterioles (402) or directly on the arteriolar muscle (2, 295), as was thought at first. In the rabbit, the pressor effect of renin is abolished or lowered by ether anesthesia (357), but this is not true of the dog.

Tigerstedt and Bergman (402) reported that renin exhibits tachyphylaxis, which means a decrease of the response of the animal to repeated intravenous injections of the same amount of renin, provided these are made only a few minutes apart. This has been confirmed by others (191, 197, 228, 240, 241, 268, 295). This effect is independent of anesthesia, type of anesthetic, type and method of preparation of renin and occurs in a bilaterally nephrectomized animal (413). It produces no direct effect on the isolated perfused heart (197, 200) but when injected intravenously into an animal, increases the force of cardiac action. When it is injected intravenously, it produces its effect even in an animal with spinal cord sectioned or destroyed (272), and its pressor effect

is not the result of the stimulation of an endocrine organ. It produces its effect in the absence of hypophysis and adrenals (272). In the rat, it is said to be effective when injected intraperitoneally, and more effective in the bilaterally nephrectomized than in the normal rat (115, 272). This should be tested on dogs and other animals. It does not produce a reduction in peripheral blood flow or a fall in the temperature of the surface of the skin (221). Its effect is not reversed by 933F (222), or ergotamine tartrate, nor is it enhanced by cocaine. It has a more prolonged vasopressor effect in bilaterally nephrectomized animals (348), probably on account of the increase of hypertensinogen in the blood of such animals. Renin injected intravenously into the normal rabbit produces diuresis and increased excretion of sodium chloride (323). It has been asserted that extracts containing renin from the kidneys of animals with experimental renal hypertension and of acutely and completely ischemic kidneys contain more renin than the extract of the opposite normal kidney, or of normal kidneys from other animals. But Landis (237) was unable to correlate the renin content of the kidney and the physiological state of the kidney. This work should be repeated with the use of the more recently devised methods of extraction and assay.

The presence of renin in the renal venous blood and systemic blood of animals with experimental renal hypertension has been inferred from the production of hypertensin, when the blood was added to hypertensinogen and incubated with it (80, 105, 298). Dexter and Haynes (82), by this indirect method, measured the amount of renin in the systemic blood of human hypertensive patients. They found renin only when the blood pressure was rising abruptly (eclampsia and acute glomerulonephritis); none in the blood of patients with benign essential hypertension. On this account they suggested that renin may be involved only initially, during the development of hypertension. The existence of renin in renal venous blood from a human kidney, the renal vein of which had been occluded for 12 minutes, has been reported (338). The criticism of most of the negative results is that too much renin is expected and therefore too small a quantity of blood has been tested. Gollan, Richardson and the author (unpublished), by incubation of larger quantities of blood, and with proper precautions to prevent the action of hypertensinase, have been able to demonstrate the formation of hypertensin by the action of renin on the natural hypertensinogen in the blood of dogs with experimental renal hypertension, both benign and malignant. In the animals, in the malignant phase, there was much more hypertensin in the final product, and therefore presumably more renin in the blood. A more direct way of determining the renin content would be to measure the rate of formation of hypertensin.

The action of renin is not affected by hypophysectomy, thyroidectomy, splanchnicectomy, gonadectomy, splenectomy, pancreatectomy, abdominal evisceration or destruction of the spinal cord (272, 278, 435). Bilateral adrenalectomy, although it has no immediate effect (272), is followed by a progressive decrease in the response to the intravenous injection of renin (210, 278). By slow intravenous administration of a small amount of renin, the blood pressure

was kept elevated about 30 mm. above normal, for four hours. Larger quantities produced untoward circulatory disturbance (201). With one exception (430), investigators agree that the effect of the purest renin so far produced is not potentiated by cocaine or reversed by ergotamine tartrate or 933F. Its ultimate effect is therefore different from that of known sympathicomimetic pressor amines. Its action differs from that of adrenalin, pituitrin and tyramine. The pressor response to an intravenous injection is more delayed because formation of the true vasoconstrictor substance, hypertensin, has to occur during this interval. The rise of blood pressure is not as steep and lasts much longer. It takes about two minutes for a unit of renin to produce its maximum rise of 30 mm. Hg direct mean pressure and the gradual return to normal may take as long as 30 minutes. The rise of blood pressure is not associated with a decrease in peripheral blood flow.

The action of the purest renin so far produced (191, 197, 228, 240, 241, 268, 395, 435, 441) is no different from that of crude renal extracts that do not also contain a depressor substance. According to Grollman and collaborators (172, 433) the amount of renin in a kidney extract, or its apparent concentration, depends upon the care of the tissue prior to its preparation, the method of preparation and the duration of time between the preparation of the extract and its assay on a test animal. The last part of the statement must apply only to very crude extracts, because the author has assayed moderately purified liquid, frozen or lyophilized preparations of renin several times during a period of three years without noting much deterioration in any of them. All preparations of renin were sterilized originally by filtration through a Seitz filter, and kept in sterile condition, without the addition of preservatives, in sealed vials. The liquid and lyophilized renins were kept at 4°C., while the frozen renin was kept at −30°C.

Fate of Renin in the Body. The finding of Tigerstedt and Bergman (402) that bilateral nephrectomy increases the sensitivity of the organism to an intravenous injection of renin has been confirmed by others (115, 272, 310, 411); but their idea that the kidney eliminates renin has not been confirmed, because it has been found that only when large quantities of renin are suddenly injected intravenously does it appear in small quantity in the urine. The maximum excretion occurs in about one hour after the injection (209). Because the absence of the liver and kidneys delays the rate of disappearance of renin injected intravenously, Leloir and collaborators (247) concluded that both play an important part in the elimination of renin from the body. The main mechanism of elimination of renin from the circulation is now considered to be destruction by the tissues of the body, including the kidney and liver (209).

The Unit of Renin. Various units have been suggested and different methods for the purification and bioassay of animal renin have been described (52, 151, 181, 191, 228, 239, 246, 267, 272, 321, 324, 329, 343, 359, 395, 410). A special method for the assay of human renin has been described (84, 280). It would be highly desirable to determine the best method, and agree upon a unit, for animal and human renin, in order to have at least one good basis for the comparison of

results obtained by different investigators. Leloir and collaborators (246) defined a unit of renin as the quantity which, when incubated with hypertensinogen in excess, at 37°C. and pH 7.5, produces 0.5 unit of hypertensin in two hours. We have used the direct method for the assay of animal renin (151) and have defined the dog unit as the quantity which, when injected intravenously into normal unanesthetized dogs, will cause a rise of direct mean pressure of 30 mm. Hg. This has proved a very useful method for purification studies (228). A method for the study of the reaction kinetics of renin has been described by Plentl and Page (325).

Hypertensinogen. This is a protein (327) of the blood plasma upon which renin acts to form the vasoconstrictor substance hypertensin (angiotonin). It is thermolabile, not ultrafiltrable, and not dialyzable. It is a pseudoglobulin (34, 278, 279, 360) and a constituent of the α_2-globulin fraction (327). Serum albumin and hemoglobin do not act as renin substrate. Edman (97), however, considers that the method and conclusions of this study by Plentl, Page and Davis (327) are "not entirely above criticism". A method for its preparation in relatively pure form, from beef serum, has been described (360). It is precipitated from serum by ammonium sulphate between 0.30 and 0.41 saturation, at pH 6.8, and does not precipitate after dialysis against distilled water.

Renin extracted from the kidneys of mammals reacts to form hypertensin with hypertensinogen from any mammal, with the exception of man. Human renin is active on the hypertensinogen of any mammal including man. Renin is found in the kidneys of chickens and ducks and it reacts with hypertensinogen from any of those birds but not with mammalian hypertensinogen. Mammalian renin is not active on avian plasma. There seems to be no renin in the kidneys of toads or sharks (14).

The reaction between renin and hypertensinogen is enzymatic and specific in the sense that only blood plasma or serum of various kinds acts as the substrate. Hemoglobin, liver, spleen, thymus, testis, lung, heart, skeletal muscle and vegetable proteins do not act as renin substrate. It requires only a small amount of renin in proportion to the amount of substrate in the blood to bring about the optimum reaction, and the amount of hypertensin formed is proportional to the amount of blood globulin. The action is not greatly affected by temperatures up to 37°C, for although it is hastened by incubation, at 37°C, yet it is only slightly retarded, and goes on to completion, at 0°C, even in the presence of hypertensinase (356). (See under Hypertensinase.) The effect of renin on hypertensinogen cannot be reproduced by pancreatin, papaine or extract of liver or spleen, nor by pepsin at the pH of the blood (see Pepsitensin).

According to Leloir and collaborators (245), the quantity of hypertensinogen is about the same in the blood of normal dogs, cows and pigs, while the horse has a little less. Lymph contains as much hypertensinogen as plasma (134). The amount of hypertensinogen is increased in the blood of bilaterally nephrectomized animals (245, 278). It is not affected by hypophysectomy. Adrenalectomized rats are less sensitive to an injection of renin (431) and hypertensinogen was found greatly decreased in bilaterally adrenalectomized

animals (138, 148, 245, 252) and in dogs with hemorrhagic shock (56), in human hepatic insufficiency (190), but not in one case of Addison's disease (189). It is not increased in the blood of hypertensive animals or in human hypertension (189). Adequate substitution therapy of adrenalectomized animals with adrenal cortical extract results in the return of hypertensinogen in the blood to the normal level. Repeated injections of renin, with final tachyphylaxis, are said to deplete the hypertensinogen in the blood (278). It is probable that hypertensinogen is formed in the liver (245). Hepatectomy and destruction of the liver by chloroform poisoning cause hypertensinogen to decrease in amount and even to disappear from the blood (148, 252, 314) and it is often significantly decreased in human hepatic insufficiency (189). Hepatectomy causes a decrease, but if bilateral nephrectomy is performed at the same time, then there is no diminution, because no renin is being liberated to use up the hypertensinogen (247).

Unit of Hypertensinogen. This may be defined as the amount of hypertensinogen which, with an excess of renin, and in the absence of hypertensinase, will produce a unit of hypertensin.

Hypertensin (*Angiotonin*). The substance formed by the action of renin on hypertensinogen is the final effector, vasoconstrictor, and therefore pressor substance of the humoral mechanism of renal hypertension which causes the increased tonus of the smooth muscle of the arterioles when renin is injected into, or presumably enters, the blood stream (279). It was discovered independently by two groups of workers who gave it the names hypertensin (36) and angiotonin (308). If it is finally proved that the product which results from the interaction of renin and the substrate in the plasma of hypertensive animals and man is the substance that causes persistently elevated blood pressure, then the term hypertensin of the South Americans (279) will be the more appropriate. It has already been shown (36) that when hypertensinogen is added to the blood plasma or serum from the blood of a totally ischemic human kidney, and the two are incubated under conditions that prevent the action of hypertensinase, a substance results which has all the properties of hypertensin (338). Evidence is accumulating that renin, (and therefore hypertensin) is present in the systemic blood of hypertensive animals and man. This is one of the most important problems that remain to be settled.

Chemical properties. Hypertensin is soluble in water, 96 per cent ethanol, 75 per cent acetone, liquid phenol, glacial acetic acid and methylene glycol, but insoluble in ethyl ether, chloroform and amyl alcohol. It is dialyzable and thermostable (resistant even to boiling temperature), alkali-labile, (destroyed completely by boiling for 1 hour at pH 10), relatively acid-stable (does not become inactivated by boiling for 2 hours in 1N HCl), can be precipitated with phosphotungstic acid, is destroyed by oxidation and acetylation with ketene (331), but is not affected by reducing agents (309). It is fluorescent and gives the color reaction for arginine, but it is not destroyed by the action of arginase (73). A positive Sakaguchi reaction is its only reaction for protein (309). It contains tyrosine and histidine, but not cysteine, tryptophane or proline (73). In

electrophoresis experiments, hypertensin behaves as an amphoteric electrolyte of neutral character (73). Cruz Coke has outlined the similarities and differences between hypertensin and the pituitary pressor hormone (73). That the reaction between renin and hypertensinogen is disintegrative is indicated by the fact that pepsin, a known proteolytic enzyme, may replace renin in this reaction, but only at a very low range of pH. Pepsin is inactive in the optimum range of pH for the activity of renin, and does not produce a pressor effect upon intravenous injection. That the reaction between renin and hypertensinogen is enzymatic is now generally accepted (325). Hypertensin is also destroyed slowly by hydrolysis, by proteases and peptidases (73). It is now considered to be a polypeptide of low molecular weight (244), although Page and Helmer (309) found that it gave a negative biuret. It is destroyed by pepsin, trypsin, papain (36, 96, 278) and extracts of some organs, as well as by fresh normal blood plasma, serum and laked blood corpuscles, all of which contain hypertensinase. The vasoconstrictor effect of hypertensin is inactivated by amino-oxidase from liver of sepia officinalis and tyrosinase from Psalliota campestris (66). It is easily destroyed during purification, for unknown reasons (244). Cruz Coke (73) lists a number of chemicals which partially or completely inactivate hypertensin, the most important being iodine. The effect of the latter is due to the transformation of the tyrosine in hypertensin. He also emphasizes the accelerated destruction of hypertensin by hypertensinase upon the addition of oxidized cytochrome C to renal extract containing hypertensinase.

Crystallization of angiotonin in the form of its picrate and oxalate has been reported by Page and Helmer (309), but this result was not confirmed (330). Recently a preparation of high potency was obtained by Edman (97). As little as 0.5 γ elicited an appreciable effect on the blood pressure of a cat. Cruz Coke (73), by means of anionic resin has been able to purify hypertensin, so that it had only 0.08 mgm. N per unit. Cationic resin adsorbed the hypertensin, but it was not possible to elute it. The melting point of this substance in pure form has not yet been reported. The effect of proteolytic enzymes on partially purified angiotonin has been investigated (330), but the authors state that "such a study cannot be taken as offering proof of the structure of this substance".

The most recent important contribution to the subject of the purification of hypertensin is by Edman (97) whose method consists of chromatography, precipitation with nitranilic acid and electrodialysis. He obtained in this way a 600–700 fold purification of the active principle, but the yield was only 3 per cent. This great loss he attributes to the lability of the hypertensin structure. His highly purified hypertensin is almost 40 times more effective in raising blood pressure than a comparable amount of tyramine phosphate. He regards his product as essentially pure, judging by its behavior on partition chromatography on filter paper. By means of electrophoresis he determined the isoelectric point to be at pH 6.8. From the diffusion constant of his hypertensin in pure water he calculated the molecular weight to be 2700. By means of paper chromatography, and some preliminary quantitative determinations, he found

in his hypertensin the amino acids lysine, histidine (28 per cent), glycine, alanine (3.8 per cent), serine, proline (5 per cent), valine, tyrosine (2 per cent), leucine (or isoleucine), aspartic acid (4.5 per cent) and glutamic acid (5 per cent).

Physiological properties of hypertensin. The pressor effect produced by an intravenous injection of hypertensin is immediate and steep, like that of adrenalin. The maximum rise from the intravenous injection of a unit of hypertensin occurs in a minute or less and the return to normal in three minutes or less. Accompanying the hypertensive effect of hypertensin, as in the case of renin, there is no change in blood flow through the skin, as measured by temperature of the skin and by direct observation of blood vessels growing in a wound in the skin of a rabbit's ear (301). There is a decrease in the volume of the spleen and kidney (36), a decrease in coronary blood flow, increase of venous pressure and decrease of renal blood flow. Hypertensin produces no significant symptoms, except forceful heart beat, when it is injected intravenously into man (301). In vagotomized dogs the heart rate is not modified (36). Continuous intravenous injection produces an increase of blood pressure which is maintained during the period of injection (36). On isolated intestinal strips hypertensin has a stimulating effect(309) and a similar effect is produced by it on nearly all smooth muscle.

Hypertensin shows no specificity. This product of the reaction between mammalian renin and hypertensinogen shows its characteristic pressor effect in dogs, chickens, toads and snakes. Dogs and chickens do not exhibit tachyphylaxis as a result of repeated injections of hypertensin. Toads and snakes are rapidly rendered tachyphylactic (14).

The assertion of Page and Helmer (301) that repeated injections of angiotonin produce tachyphylaxis by the method of perfusion of the rabbit's ear has not been substantiated by the work of others (278) with the repeated injection *in vivo*. The method of perfusion of the rabbit's ear is notably unreliable and of questionable value in this connection. Existence of angiotonin activator, postulated by Page (301), was not confirmed. As in the case of renin, injected intravenously, the pressor effect of hypertensin is unaffected by cocaine (191,309), atropine (309), ergotamine (191), 933F, or stilbestrol (309). It is therefore not a sympathicomimetic substance. The effect is greatly increased in bilaterally nephrectomized dogs. Hypertensin, like renin, when injected intravenously, induces a pronounced but transient rise in potassium and a greater and more prolonged rise of sugar in the blood of dogs under the influence of chloralose. No special significance has been attached to these observations and they have not been confirmed (113). The striking difference between renin and hypertensin is that hypertensin produces vasoconstriction when it is added to Ringer's solution and perfused through an isolated organ from which the blood has been washed out, while renin in Ringer's does not have this effect. The direct effect of hypertensin and the indirect effect of renin are exerted on the musculature of the peripheral blood vessels. Large doses of hypertensin injected intravenously into normal persons caused a decrease in blood volume and cardiac output, as measured by the ballisto-cardiograph (400). The effect

of hypertensin injected intravenously is not affected by vagotomy, excision of the carotid bodies, splanchnic nerves, pituitary, pancreas, liver or adrenals, by evisceration, destruction of the adrenal medulla or rapid elimination of the central nervous system, in cats and dogs (97, 307).

The finding of Page and collaborators (233, 295, 298, 299, 300, 302, 304) that there is in the peripheral systemic blood of hypertensive animals and man a vasoconstrictor "angiotonin-like substance", not present in the blood of normotensives, has not been confirmed. There is no proof that this hypothetical substance is identical with renin or hypertensin. These studies were also carried out by the rabbit's ear perfusion method, so the results will require confirmation by some other method before they can be accepted. The same holds true for the demonstration of a pressor substance in blood perfused through an isolated kidney with renal artery constricted, demonstrated by the same method (233).

Gregory and collaborators (162) have failed to demonstrate an increase of vasoconstrictor substance for frogs in the ultrafiltrate of blood plasma of human beings with essential hypertension of long duration. Basing themselves on the results of a quantitative method of questionable value, they concluded, without full justification, that essential hypertension is probably not caused by an increased production of hypertensin. Dexter and Haynes (82) failed to produce hypertensin when they added hypertensinogen to the systemic blood of patients with essential hypertension. For the present, at least, the most obvious criticism of this work is that the amount of blood tested has always been too small. More studies of larger amounts of blood are necessary. The failure of Grollman and Rule (177) to demonstrate a pressor effect in the normal rat of parabiotic twins, of which one was hypertensive, on a renal basis, does not disprove the existence of a humoral mechanism in this type of hypertension and does not prove their contention that the normal kidney elaborates a substance the absence of which results in hypertension. The explanation of their results may be simply the slow passage of the hypertensin from one animal to the other and the rapid destruction of that which does pass across (31).

Unit of Hypertensin. This is best defined as the quantity of hypertensin which, when injected intravenously in the unanesthetized trained dog, will give a rise of 30 mm. Hg direct mean femoral blood pressure. Braun-Menendez and collaborators (36) consider an elevation of 20–30 mm. as a unit, because of a greater variability of response to the same dose than we have noted. This difference may be due to the fact that our animals are well trained and lie quietly during a determination. This is a necessary condition for uniform results from injections of renin or hypertensin. They have published a curve of the pressor effects of various amounts of hypertensin from which the number of units is estimated by interpolation (36). This is not accurate, in our experience, since the pressor response to hypertensin is not a linear function of the concentration of hypertensin. It would be highly desirable to agree upon one method.

Pepsitensin. It has been shown by Croxatto and collaborators (68) that pepsin, if it acts on blood globulin, at pH 3.0, or lower, can produce, by peptic

digestion of the protein, a vasoconstrictor and pressor substance which they have called pepsitensin and which they assert is identical in chemical and pharmacological properties with hypertensin, except that pepsitensin is not inactivated as rapidly by hypertensinase (6, 37). This difference is attributed to impurities in pepsitensin and it is asserted that the purer the pepsitensin the more sensitive it is to inactivation by hypertensinase and other proteolytic enzymes (73). Hypertensin is more easily destroyed than pepsitensin by the action of pepsin. This is to be expected, since pepsitensin results from the action of pepsin on blood globulin. Renin and pepsin, however, do not act on exactly the same substrates. Plasma globulins acidified to pH 2 and then neutralized, or precipitated with alcohol and redissolved, no longer yield hypertensin with renin, but still yield pepsitensin with pepsin. It would seem that pepsin acting on denatured globulin is capable of producing a pressor substance, while renin fails to do it (244). The existence of pepsitensin has been confirmed by others (192, 424), including ourselves. What significance these experiments will have eventually in connection with the humoral mechanism of renal hypertension it is difficult to estimate at this time. For the present they merely indicate the probable non-specific character of the renin-hypertensinogen reaction, and the disintegrative nature of this reaction, which results in the formation of an active pressor substance.

Hypertensinase. It was noted by Page and Helmer (310) that when renin was incubated with plasma or serum, angiotonin was formed, but that continued incubation resulted in the destruction of the angiotonin. They attributed this to the effect of the renin. Muñoz and collaborators (278), however, showed that this inactivating effect of the renin could be eliminated while the capacity of the renin to produce the pressor substance remained unaffected. This led them to postulate the existence of another enzyme associated with impure renin. For this enzyme the name hypertensinase was chosen by the South Americans (107), and it has been called angiotonase, to correspond with angiotonin, by Page and collaborators (313).

Hypertensinase is a hydrolyzing enzyme, or group of enzymes (107, 278), present in blood plasma and serum, in laked red blood corpuscles, and in extract of organs, especially intestine, kidney, pancreas, spleen and liver, with the ability to destroy hypertensin *in vitro*. Intestinal mucosa is the richest source of this enzyme, while blood serum and plasma that are not hemolyzed contain only a relatively small amount (107). There is no increase of hypertensinase in hypertensive dogs (81) or man (190).

Properties. Hypertensinase is a protein and an enzyme, or mixture of enzymes, not dialyzable, and precipitable by half saturation with ammonium sulphate. It is acid and heat labile (destroyed at 60°C. or over). The activity of hypertensinase is optimum at a pH between 7.5 and 8.5. This enzyme is inactivated, if kept at pH 3.9 for 15–20 minutes, at 37°C., even in the presence of renin and hypertensinogen. The action of hypertensinase is greatly decreased at 18°C. (13) and becomes negligible at O°C. (356), at which temperature the reaction between renin and hypertensinogen is only slightly retarded, but may

go on to completion. Although hypertensinase has not yet been isolated in pure form, Croxatto and collaborators (69, 70, 71) have concluded that its effect on hypertensin is due to a peptidase, and most probably an aminopeptidase action (72). This agrees with the finding that the hypertensin inactivating effect of an extract of pancreas is due to carboxypeptidase (330). Their conclusion, on the basis of inhibition experiments, is that the hypertensinase effect of renal extract and of laked red blood corpuscles is partly proteolytic and partly an oxidase effect. From renal extracts it is difficult to obtain hypertensinase free of renin, but the hypertensinase may be easily destroyed in a solution of renin. There is some probability that the kidney may be the main source of the hypertensinase in normal blood plasma. This is indicated by the fact that there is almost complete absence of hypertensinase in the blood of bilaterally nephrectomized dogs. There is less hypertensinase in lymph than in plasma (134). The fact that the blood from the ischemic kidney contains less hypertensinase than normal blood may mean that the ischemic kidney produces less (426). The exact part, if any, played by hypertensinase in the maintenance of normal or elevated blood pressure is not yet elucidated.

The Unit of Hypertensinase. This has been defined as the smallest amount of hypertensinase which is capable of inactivating one unit of hypertensin in 30 minutes at 37.5°C. The technique for the determination of this unit is to combine variable amounts of hypertensinase with a constant amount of solution known to contain one unit of hypertensin. The mixture is incubated for thirty minutes at 37–38°C. and is then injected intravenously into a normal, unanesthetized trained dog. The least amount of hypertensinase which destroys the entire unit of hypertensin, so that there is no rise of blood pressure after its intravenous injection into an animal, is the unit of hypertensinase (107).

Mechanism and Site of Formation or Release of Renin. Despite the vast amount of work that has been done on the properties of the various constituents of the humoral mechanism of experimental renal hypertension, but little of a definite nature has been established beyond question about the mechanism and site of formation and release of renin, upon the action of which the formation of hypertensin depends. Although Leloir (244) states unequivocally that most of the known facts about the mechanism of action of renin are consistent with its being the cause of renal hypertension, yet Grollman (166) even questions the existence of preformed renin in the kidney, and considers that it is merely the product of autolysis *in vitro*. His experiments may merely indicate that in the living animal a renin precursor, *prorenin*, as it leaves the living cell, undergoes a change which transforms it into renin, and that the same transformation can also occur *in vitro*. Analogous transformations are known to occur in proteolytic enzymes; for example, the formation of trypsin or pepsin from their inactive precursors.

Exactly what takes place in the kidney, with main renal artery constricted adequately to give hypertension, without causing obvious degeneration or necrosis in the substance of the kidney, is not well known. The observation of decreased oxygen consumption by the ischemic kidney (249) or by ischemic renal tissue (140) has been confirmed (339); but the result has been questioned

on the ground that the reduction may be due to death of a certain number of cells and not to interference with the function of all (264). Inhalation of 7–10 per cent O_2 did not cause a greater rise of the blood pressure of hypertensive dogs (390), and continuous inhalation of 100 per cent oxygen for 48 hours failed to lower their blood pressure. This has been interpreted as not being in favor of a hypothetical anoxemic factor in the pathogenesis of experimental renal hypertension. Yet Cruz Coke (73) has arrived at the view that tissue anoxia, especially renal, plays an important part in the humoral mechanism of renal hypertension. The criticism of the experiments on oxygen consumption may also possibly apply to the demonstration of a great diminution of the cytochrome C concentration and the activities of the cytochrome oxidase and succinic dehydrogenase in slices and homogenates of the kidneys of hypertensive dogs (340). More work of this kind must be done before its significance can be evaluated.

The site of origin of renin, at least *in vitro*, has been investigated, and the evidence at the present time is that it originates in the cortex of the kidney and especially in the lining epithelium of the convoluted tubules. The fact that extract of aglomerular midshipman fish kidney contains no renin (130) has provided no clue to the origin of renin because it has been found that marine fish kidneys which do possess glomeruli do not contain renin, while the kidneys of fresh water fish possess it in great abundance. The explanation for this difference has not been found (132). Yet renin can be produced from the kidney of the dolphin, a marine mammal (98). Because the renin content of the involuting tubular portion of the mesonephros of pig embryo decreased, while that of the developing tubular portion of the metanephros increased, the conclusion was reached that the convoluted tubules are most probably the site of origin (production and storage) of renin (220). In keeping with this is the failure to extract renin from kidneys in which the proximal convoluted tubules had been destroyed by sodium tartrate poisoning (131).

The nature of the stimulus for the release of renin has not yet been determined. The idea that reduction of intrarenal pulse pressure, rather than decreased blood flow to the kidney, is what determines the release of renin and the formation of the vasoconstrictor substance, depends entirely upon the demonstration of a pressor substance in the blood by the rabbit's ear perfusion method; and since, by admission, this is not really a test for renin or angiotonin, it is questionable what significance can be attached to these experiments (62). The assumption of a presumable change from intermittent to continuous pressure beyond the site of the afferent preglomerular arterioles is not justified, for the very reason that a pulsatile pressure in the glomerulus has never been proved to exist. Braun-Menendez (31) states unequivocally that, "The idea that diminished pulse pressure within the kidney causes the liberation of renin has no solid experimental proof". The reduction of blood flow through the functioning components of the kidney (glomerular and peritubular capillaries) is another possible stimulus (152, 250). That there is a reduction in the blood flow through the kidney in most cases of essential hypertension, affecting both kidneys equally (47, 48, 63, 155, 364), as well as in the early stage of experimental renal hypertension (152),

is an established fact, but there is still a question about whether permanent reduction of the blood flow is necessary for the persistence of hypertension in animals (369). This must await better and more direct methods than are available at present to allow frequent determinations of renal blood flow before and after constriction of the renal arteries. Because, in an occasional animal, there was no permanent reduction in the blood flow through the kidney, although there was still a slight increase in blood pressure, some authors (61, 62) have brushed aside the idea that some reduction of blood flow is a necessary condition for the development of the hypertension. For the demonstration of true renal ischemia Chasis and Redish (48) require that the ratio of renal plasma flow (diodrast clearance) to tubular excretory mass (maximum tubular secretion of diodrast) should be calculated, because the reduction of diodrast clearance does not necessarily mean renal ischemia. Smith and collaborators (153, 155, 383) state that the evidence favors the view that the renal ischemia, so frequently observed in essential human hypertension, is a secondary event, and that the primary event is the circulation of a humoral substance of unknown origin which brings about the efferent arteriolar spasm and progressive and parallel reduction in renal blood flow, which they consider characteristic of essential hypertension Others believe that the efferent arteriolar spasm is due to the angiotonin produced by renin in the blood (193), but in experimental renal hypertension this begins only after the renal artery is constricted, and therefore, in man, it should begin only when the renal arteries and afferent arterioles are sufficiently diseased to reduce the size of the lumen.

The demonstration (65) *in vitro* of a normally perfusable vascular bed in the kidneys of human beings with benign hypertension, especially when the perfusing fluid is kerosene, certainly does not justify the conclusion that the perfusion of blood through the kidney *in vivo* is also normal in such individuals. It is interesting, however, that despite the obvious objection to the method, there was a great decrease of the rate of perfusion, even of kerosene, through the kidneys of patients with uremia, due to arteriolosclerosis, glomerulonephritis or pyelonephritis.

The problem of the possible part played by the juxtaglomerular apparatus in the humoral mechanism of experimental renal (156, 157) and human hypertension (229) is by no means settled. This apparatus has been described in detail by Goormaghtigh (156, 158 158a) and others (94, 95, 269).

An increase in the size of the "polkissen", or juxtaglomerular apparatus, and in the number and size of the afibrillar, and sometimes granular, or vacuolated, cells in this apparatus, in the kidneys of dogs with renal hypertension, produced by constriction of the main renal arteries, has been described (158). Goormaghtigh (158[a]) considers that these cells may have a local, or even general, secretory, or humoral activity and may therefore have a direct relationship to the hypertensive principle. In the rabbit, in which afibrillar cells are common in the normal kidney, Dunihue (95) states that Goormaghtigh has found an increase in the number of afibrillar and granular cells in the juxtaglomerular apparatus of rabbits made hypertensive by constriction of the main renal arteries. Goormagh-

tigh considers that the afibrillar cells have to do with arteriolar tone and that the granular cells are a source of the pressor substance. These findings in the hypertensive rabbit have been confirmed by Dunihue (94, 95) who subscribes to the same views. Kaufman (229), on the basis of an anatomical study of kidneys from normal and hypertensive persons, reached a similar conclusion. Graef and Smith (160, 382), however, have drawn attention to the great variation in the appearance of the arteriolar media and the size and structure of the juxtaglomerular apparatus in normal man and animals and have cautioned that, because of species differences, e.g., the absence of granular cells in the kidney of man and dog, the interpretation of the ischemic changes must be contingent upon a more complete study of normal kidneys. The development of cytologic changes in the juxtaglomerular apparatus, interpreted by some investigators as indicative of endocrine activity, does not constitute convincing proof of the origin of an endocrinogenic pressor substance or precursor in this structure.

There is certainly no direct, convincing evidence that any special cells in the juxtaglomerular apparatus or preglomerular arterioles are the source of a chemical factor which constricts the afferent or efferent arterioles, thus regulating glomerular blood flow, and, presumably, also the peripheral arterioles, with resultant systemic hypertension. As a matter of fact, the presence of renin in the developing pig embryo, in which a definite juxtaglomerular apparatus has not been identified, militates against this view (220). More investigation is is necessary before the functional significance of the juxtaglomerular apparatus can be properly assessed.

The Treatment of Experimental Renal Hypertension by Renal Extracts. After the production of experimental renal hypertension was accomplished, it was hoped that methods of treatment which would be successful for hypertension in animals might be applied with equal success to human hypertension. It was soon shown that all the methods which had previously failed to affect human hypertension were equally unsuccessful in experimental renal hypertension (150, 173), but that the difficulties involved in the clinical appreciation of the hypotensive properties of various agents in essential hypertension are greater than in experimental renal hypertension (4).

Any attempt to treat experimental renal hypertension by affecting the humoral mechanism should take into consideration the following possibilities outlined by Muñoz and collaborators (278): 1, suppression, diminution or inactivation of renin; 2, inhibition of the reaction between renin and hypertensinogen; 3, diminution of the amount of hypertensinogen; 4, inhibition of the action, or destruction, of hypertensin, by an increase in the amount or activity of hypertensinase, or some other agent capable of accomplishing this. Methods 1 and 4 have received greatest attention. The fourth method will be discussed first.

Extracts of kidney had been used empirically, with variable results, for the treatment of human hypertension (150) a long time before persistent hypertension was produced in animals. The first attempts to treat experimental renal hypertension by means of renal (and muscle) extracts were made by Harrison and collaborators (174, 175, 176, 179, 180, 185) who reported a lowering

of blood pressure in hypertensive rats by renal extract given by mouth, or parenterally. Their attempt was based mainly on the assumption (179) that in the temporary hypertension due to unilateral constriction of the main renal artery, the normal kidney, by some humoral mechanism, e.g., by inhibiting or destroying the pressor substance, might be playing a part in eliminating the hypertensive effect of the kidney with renal artery constricted. Other evidence for this view is as follows: the removal of a normal kidney in the presence of one with renal artery constricted causes the blood pressure to remain permanently elevated; after the removal of the ischemic kidney, if the other is normal, the blood pressure falls to normal in six hours, but it takes five times as long for the blood pressure to reach normal after the removal of both kidneys, when one or both are ischemic. This led Katz and collaborators (223, 226, 349) and others (106, 404, 405) to the idea that by counteracting or neutralizing the pressor substance the normal kidney might play a part in the elimination of the chemical mediator of experimental renal hypertension. They believed that the more rapid fall of the blood pressure to the normal level, in the presence of normal kidney tissue, as contrasted to the slow fall in its absence, might be due to the excretion, or destruction *in vivo*, of the pressor substance, by the remaining normal kidney. They showed that in an animal with a ureteral-venous fistula on the side of the normal kidney, the removal of the opposite ischemic kidney was still followed by rapid (6 hours) fall of the blood pressure to normal. They concluded that the normal renal tissue destroyed the pressor substance, since otherwise its continued presence in the bloodstream should have kept the blood pressure elevated for a longer period. Dexter and Braun-Menendez (78) have demonstrated that the renal threshold for the excretion of renin is too high to account for the stabilizing rôle of the normal kidney. Subsequent studies by Grollman and collaborators (166) have led them to a modification of their original view and the adoption of the idea that the active principle derived from kidney tissue is an essential humoral agent, the absence of which, in the diseased kidney, results in hypertension. They now believe that the administration of this principle overcomes the deficiency and relieves the hypertension.

The demonstration that renal vein blood from an ischemic dog kidney (129) contains much less hypertensinase than renal vein blood from normal kidney has thrown light on the earlier experiments of Freeman (116) who found that normal dog's blood can reduce the blood pressure of dogs with experimental renal hypertension, whereas blood from hypertensive dogs has no such effect. The antipressor effect in this case may have been due to the presence of hypertensinase in normal blood and its diminution in the blood of hypertensive animals. These experiments require confirmation.

Page and collaborators (297, 311, 312, 313) and others who have used identical extracts (123, 281) have reported the reduction of arterial blood pressure of hypertensive animals and patients by the parenteral (intramuscular) administration of renal extract which contains a substance capable of inhibiting the pressor effect of angiotonin (59). Although Page and collaborators (313) have tried

to relate the antipressor effect of their kidney extract to the hypertensinase content, yet there is little hypertensinase in it, and Schales and collaborators (361) found that this extract gave the same result when its hypertensinase was first destroyed. Besides, there is no proof that hypertensinase *per se* is absorbed into the blood from an intramuscular injection.

It has been shown that for the antipressor effect of the renal extracts used for the treatment of hypertension, hypertensinase is not required. Harrison, Grollman and Williams (185) did not determine the existence of hypertensinase in their extract, but a lowering of blood pressure has been reported in the rat, dog and even man by the administration of renal extract both orally and intramuscularly. That such effects are caused by renin inhibitor, as postulated by Page and collaborators, has not been confirmed.

The possibility that all the effects on blood pressure by renal extracts, hitherto reported, are due to a non-specific pyrogenic effect (with or without actual elevation of temperature) of a foreign organic material that produces a local and general reaction, has already been suggested (46, 150). Indeed a reduction in the severity of the local reaction, as a result of purification, has resulted in a reduction of the antipressor effect. Schales, Stead and Warren (361) observed an antipressor effect which was not specific for renal extracts, and attributed all the effects they observed to the local and general reactions which occurred. They believe that this invalidates any conclusions about the specific effect of renal extracts. The same effect on blood pressure was obtained when the extract contained little or no hypertensinase, if the local and systemic reactions to the injections occurred. The extract that Grollman, Harrison and Williams used can hardly be the same as that of other investigators, because they found that the active substance was dialyzable and effective by mouth in both man and animals (174, 179, 180, 432). The experimental work on animals was all done on the rat, and has not been confirmed. No other investigators have reported a lowering of blood pressure in hypertensive individuals from the oral administration of renal extracts. Goldring and Chasis (153) failed to observe any favorable effect in four hypertensive patients treated with renal extract, given by mouth, up to the equivalent of 50 kgm. of original kidney substance daily.

Page and collaborators insist that they have observed fall of blood pressure from renal extract, without accompanying local or systemic reaction. However, it has been shown that all the other effects, except elevation of temperature, can be produced by the parenteral injection of foreign organic substances (46, 150, 153). The fact that there has been but little progress in the treatment of hypertension by renal extracts during the past 6 years is in itself an indication of the difficulties involved, and possibly of the inadequacy of the method.

Antirenin: Treatment of Hypertension by Means of Parenteral Injections of Renin. Because renin is a protein, it was to be expected that it would be antigenic and that parenteral injection of it would result in the development of an antibody in the blood plasma. What the properties of such an antiserum would be could not be anticipated; but it was hoped that it would prove to be antipressor, that is, antirenin.

In a series of studies, Wakerlin and collaborators (217, 414, 418, 419) reported that in the serum of rabbits, dogs and guinea pigs, but not the horse, injected with renin from various species, a substance or principle developed which neutralized the acute pressor effect of an intravenous injection of renin. They found that if the antiserum was first mixed with the renin and kept for about 18 hours at about 4°C, the mixture lost its pressor effect on the normal dog. Normal serum did not possess this property. The author and collaborators (223a) found subsequently that at room temperature the reaction between the antiserum and renin occurs practically instantaneously and that incubation for 18 hours is not required. Wakerlin and collaborators regard this principle as analogous to an antibody, antienzyme or antihormone, and suggested for it the name antirenin. This has now been produced in the rabbit, by the intramuscular injection of human, hog, cat and dog renin; in the guinea pig, by the intra-abdominal injection of hog, cat and dog renin, and in normal and renal hypertensive dogs, by the intramuscular injection of hog and rabbit renin. The injection of heat inactivated homologous or heterologous renin did not induce the formation of antirenin in either normal or hypertensive dogs (216, 415). Hog liver extract failed to produce antirenin in normal or hypertensive dogs.

Antirenin is a protein, but it is neither an enzyme nor a precipitin. Even when the antirenin titer is high, there is no precipitin in the blood of dogs or guinea pigs. In the rabbit, in which precipitin responses are more easily obtained, precipitins against other plasma proteins are formed in high titer when these animals are given repeated intravenous injections of renin. The serum of these animals still retains its ability to neutralize renin when the precipitin is removed by absorption *in vitro*. Antirenin is precipitated from serum by half saturation with ammonium sulphate. Antirenin is present in the pseudoglobulin fraction of the serum, where the circulating antibodies are ordinarily found. This fraction was found to contain practically all the antirenin of the original serum. It is active between a pH of 2.3 to 10.7 but is inactivated at a pH of 1.9 or less and 11.0 or more. At 0°C. and in the frozen state it remains stable for at least 3 months (223a). Antirenin does not neutralize the pressor effect of either hypertensin (217) or pituitrin (216). Like antihormones, (401) antirenin is not species specific in the neutralization of renin, *in vitro*, except for antirenin from rabbits induced by the injection of human renin. This specificity is also true for antihormones produced in rabbits by the injection of extracts from human tissue. Heating of the antiserum at 65°C for 10 minutes does not inactivate the antirenin, but the latter is destroyed by heating at 75°C. or higher (223a).

Wakerlin and collaborators were the first to report a fall of blood pressure to normal as a result of the repeated intramuscular injection of hog renin in hypertensive dogs. They also observed the prevention of hypertension in dogs that received similar injections before the renal arteries were constricted (415, 416). Both of these observations we have confirmed (223a). They found that the intraperitoneal injection of 40 ml. of dog serum containing a high titer of antirenin to hog renin had no specific effect on the blood pressure of one hypertensive dog.

No significant changes have been observed in the animals that have developed high titers of antirenin in their plasma. After a normal or hypertensive animal has developed a high titer of antirenin, large doses of homologous or heterologous renin may be injected intravenously into them without producing any change in the blood pressure. We have injected intravenously, in one dose, as much as 100 dog units of hog renin, without any elevation of the blood pressure, in a dog with 10 units of antirenin per cubic centimeter of its blood serum. The kidneys of rabbits and dogs which had a high titer of antirenin in the blood were found to contain normal amounts of renin (217).

Winternitz and collaborators (440) failed to confirm the production of antirenin, and Friedman and collaborators (133) did not observe a fall of blood pressure in animals that received parenteral injections of renin. But the former injected the renin intravenously and the latter probably injected an insufficient amount. The author and collaborators also failed to detect the production of antirenin in dogs by the intravenous injection of 25 units of hog renin a day, for 12 weeks, but, by subcutaneous and intramuscular injection of the same amount of hog renin, we did succeed in producing antirenin of high titer in the plasma of normal and hypertensive dogs. We also observed a fall of blood pressure in hypertensive dogs and prevented the development of hypertension in dogs by repeated subcutaneous injections of hog renin before the renal arteries were constricted. The optimum dose was 25 units of renin daily.

Wakerlin and collaborators were the first to produce antirenin, yet they now consider that they have good evidence that the antirenin is not responsible for the prevention of hypertension or the lowering of the blood pressure in dogs with renal hypertension. The evidence upon which they repudiate the significance of the antirenin is not conclusive. One reason given is that highly purified hog renin was as effective as partially purified hog renin in stimulating the formation of antirenin, but that it was much less effective as an antihypertensive agent. It is highly probable, however, that this difference was due only to the smaller amount of renin injected. Another reason was that in an occasional dog injected with hog renin inactivated by heat, a fall in blood pressure was noted. Not too much significance should be attached to this, because in some untreated dogs the blood pressure also returns to a lower level. They also found no correlation between the antirenin titer and the antihypertensive effect. This is contrary to the results of our own studies. Because they did not give an exact determination of the titer of antirenin attained by their animals, it is difficult to make a direct comparison between their results and ours. The methods of assay of antirenin are not comparable. Their antirenin titer is based on the amount of renin extracted per gram of renal cortex, which is obviously subject to considerable variation. We titrate the exact amount of antirenin by determining the number of units of renin inactivated by the serum. Our dog unit of antirenin is the minimum quantity which neutralizes the acute pressor effect of 1 dog unit of renin.

The possible application of the results obtained in animals to the treatment of human hypertension is beset with difficulties. Because homologous renin

does not produce antirenin, the injection of human renin into hypertensive patients would be ineffective. Whether heterologous animal renins which do not produce a pressor effect in man would induce the formation of antirenin is still unknown. Much more work on this subject is required. It has not been given adequate attention by other investigators.

Non-specific Pressor and Antipressor Effects. In any consideration of the renal humoral mechanism, mention must be made of the studies by Bing and collaborators (19, 20, 21) and others (205) which, although they may have no direct bearing on this mechanism, yet indicate a possible way whereby a pressor substance of renal origin may be formed, or released, under conditions which involve a disturbance of intrarenal hemodynamics. They demonstrated (20) by perfusion experiments that an ischemic kidney, but not a normal one, is capable, by decarboxylation, of converting dihydroxyphenylalanine (dopa), a substance which possesses no pressor properties, into hydroxytyramine, which is a powerful pressor amine. They showed that the amount of hydroxytyramine which is formed in the kidney from l-dopa under conditions of oxygen lack was proportional to the reduction of blood flow through the perfused kidney (19). They also demonstrated (19) the transformation of dopa into hydroxytyramine in kidney extract under conditions of low oxygen tension. But even without the addition of l-dopa the development of a pressor substance has been reported merely as a result of anaerobic conditions (407). Liver, spleen, lung and heart treated the same way did not develop pressor substances. Liver and intestine also contain l-dopa decarboxylase, yet these organs were unable, even when their circulation was reduced, to produce hydroxytyramine from l-dopa which was added to the blood (19).

Bing was also able to show that partly or completely ischemic cat's kidneys, *in vivo*, converted l-dopa, injected intravenously, into hydroxytyramine. The observation of Oster and Soskin (291) that the intravenous injection of l-dopa into cats with experimental renal hypertension resulted in a great rise of blood pressure, while no rise occurred in normal cats, is of interest in this connection. But Bing and collaborators did not conclude that hydroxytyramine is the cause of, or in any way directly involved in, the pathogenesis of experimental renal or human hypertension. The same conclusion has been reached about tyramine (100, 347, 404). Bing and collaborators have demonstrated that the two pressor substances, hydroxytyramine and hypertensin, are destroyed by different fractions of renal extract and by different mechanisms. It has also been shown that renin does not effect the decarboxylation of l-dopa to convert it to hydroxytyramine (67). It has been demonstrated that the reduction of the blood flow to the kidney results in a profound alteration of the subsequent chemical events. The kidney has the capability of converting the amino acid, which is itself without pressor property, into a powerful pressor amine which accumulates under anaerobic conditions and results in elevation of blood pressure. Pressor amines are rapidly destroyed, however, by oxidative enzymes when the circulation is normal and aerobic conditions prevail. Their work has provided a basis for the hypothesis that the hypertension which results from renal ischemia may be due to

diminished deamination of certain amino acids in the kidney and that it may possibly play an adjuvant or even an important primary part in hypertension in some types of animals.

The work of Bing and collaborators has been the basis of the attempts (127, 290, 386) at the treatment of experimental renal hypertension in rats by means of the quinones, and, in hypertensive dogs, by a diketone, 1-4-cyclohexandione (288). A lowering of blood pressure was reported, but we have not been able to confirm any of these results by the use of these materials in hypertensive dogs.

Pressor amines certainly exist and produce their effect when injected intravenously into animals. Their deamination requires oxygen. The question is whether some disturbance in the kidney may so reduce the available oxygen that deamination of natural amines does not occur and that the entrance of such amines into the blood stream results in hypertension. There is, as yet, no available evidence from the studies on man that this mechanism plays a part in human hypertension. However, the studies on the treatment of human hypertension with amine oxidases (365) has not led to an elucidation of this problem. Schroeder and collaborators (362, 366, 367) found that the parenteral injection of tyrosinase, a phenol oxidase, reduced the blood pressure in rats and dogs with experimental renal hypertension, and in man, with essential hypertension. But Prinzmetal and collaborators (332) showed that the effect obtained by Schroeder was not due to the enzymatic acitvity of the tyrosinase, because the extract containing it was also effective when the tyrosinase activity was first destroyed.

The observation that both hypertensin and pepsitensin are inactivated by an amino-peptidase obtained from yeast has led Croxatto and Croxatto (69) to conclude that the hypertensinase activity of renal extract may also be attributed to the enzyme amino-peptidase contained in renal tissue. They have also shown (66) that the vasoconstrictor effect of hypertensin tested on the perfused toad is destroyed enzymatically by the action of amine oxidase and tyrosinase. In our own laboratory, in collaboration with Gollan and Richardson, much work has been done on enzymes, from many plants that are capable of inactivating hypertensin *in vitro*. It is difficult to make preparations that are not toxic, and although antipressor effects have been obtained in hypertensive dogs, yet it is difficult to estimate how much of the effect was due specifically to the "plant hypertensinase".

The problem whether there is a renal mechanism of hypertension which is dependent upon a metabolic fault in the kidney, interfering with the utilization of melanin-like substances and their phenolic precursors, is not yet settled. Even the possibility that such a mechanism exists in one animal, the rat, for example, and not in other animals, including man, has not been determined with certainty.

The effect of diet on experimental renal hypertension has not been exhaustively investigated. It has been asserted that a high protein (800 g. meat daily) diet, or 50 g. of urea daily, increases the elevated blood pressure in dogs with experimental renal hypertension (259). A diet that produces considerable gain of weight has a similar effect (43).

The significance of the experiments of Calder (40, 41) on the production of hypertension in the rat by a diet deficient in the heat stable fraction of the vitamin B complex is of interest. His belief is that the hypertension is of metabolic origin and due to diminished oxidative processes in the kidney. This work requires confirmation.

The claim for the effectiveness of ascorbic acid in reducing the blood pressure of hypertension in man (75) has not been confirmed (276). This has not been tested on hypertensive animals. It has been reported that vitamin E has no effect on the blood pressure of dogs with experimental renal hypertension (276). This has not been tested on hypertension in man. The effect of a special rice diet (231, 232) in lowering the blood pressure of human hypertensives has not been substantiated, and the mechanism of its action is not clear. This has not been tested on hypertensive animals. The assertion that large doses of vitamin A lower the blood pressure of hypertensive human beings (159), rats (169) and dogs (420, 421) has not been substantiated by the same investigators (159, 168, 417), who finally decided that it was not the vitamin A that produced the effect (164, 168, 275, 276, 417) because fish body and liver oil still contained the blood pressure reducing substance when the vitamin A was destroyed. In fact, Grollman and collaborators (417) found that a highly purified vitamin A concentrate did not have the hypotensive effect and that the effect was enhanced by the oxidation of the fatty acid in effective marine oil. The nature and mechanism of the action of this substance in marine oils is still unknown.

In his most recent publication on this subject, Grollman (165) asserts that a number of refined oils derived from marine fishes, when administered orally, reduce blood pressure in hypertensive rats, dogs and man. Tung oil, but no other oils of vegetable or animal origin, was also effective in hypertensive animals. Oxidation, and saponification prior to oxidation, enhanced the activity of some of the oils. The effect was independent of their original vitamin A content and was retained after the destruction of this vitamin. The active principle is soluble in water and dialyzable, as in the case of the orally effective renal extracts (185, 187) with which he is inclined to consider this identical. He states, however, that it is still impractical to treat patients because there is no readily available source of large amounts of this principle.

It has been reported (282) that vitamin A may affect urea and inulin clearances, but these authors did not control their experiments by giving the same product with the vitamin A inactivated. Vitamin D_2 given by mouth had no effect on the blood pressure of the rat (38). Another sterol that has no effect on experimental renal hypertension in the dog is testosterone (412).

On the basis of experiments on hypertensive rats, a disequilibrium in sodium balance was noted by Grollman and collaborators (167, 170, 171) which led them to test the effect of restriction of sodium intake on hypertension in the rat and in man. They noted a lowering of blood pressure in both. The significance of these observations remains to be elucidated.

The most recent contribution by Shorr, Zweifach and Furchgott (380) on the humoral mechanism of shock is of great interest, but the results must await confirmation and elucidation before they can be evaluated. The identity of the

vaso-excitor (VEM) and vaso-depressor (VDM) substances which they have described, with the known constituents of the humoral mechanism of renal hypertension, cannot be considered as established on the basis of the evidence submitted. The possible part these substances may play in the maintenance of blood pressure at normal and elevated levels cannot yet be determined from the resuls that have been published.

A Summary of the Similarities Between Human Essential and Experimental Renal Hypertension. Although Goldring and Chasis in their excellent book (153) conclude that the weight of the evidence is against the identity of the mechanism in human and experimental renal hypertension, yet it has been shown by many investigators that the latter does faithfully reproduce human essential hypertension in many respects. In both, the increased tension is the result of a generalized increase of peripheral vascular resistance of functional (vasospastic) origin. In experimental renal hypertension, as in human hypertension, there may be no significant disturbance of renal excretory function (the benign phase) or there may be pronounced renal excretory functional disturbance, with uremia (the malignant phase), depending entirely upon the degree of constriction of the main renal arteries (144). As in human hypertension, so also in experimental renal hypertension, cardiac action is increased (258), but cardiac rate, output (204), volume (15), viscosity (297) and peripheral flow of the blood (221), and venous pressure remain unaltered. Pulmonic arterial pressure is not altered in either man or animals, when the hypertension is uncomplicated by left ventricular failure (227, 391), as indicated by a normal right heart. In both man and animals hypertension associated with unilateral renal disease may be cured by excision of the diseased kidney, provided the other is normal (252). Bilateral nephrectomy does not result in a rise in blood pressure in either man or animal (144). With few exceptions, the response to medicinal therapeutic measures of great variety is the same in both. Sympathectomy, partial or extensive, may result in at least a temporary fall of blood pressure in human hypertension, yet it does not do so by affecting the primary cause of the hypertension; but there is little or no effect in animals that do not stand erect. Whether this difference is significant cannot be stated with certainty at present. The fact that, after sympathectomy, the blood pressure returns to the original high level in a high percentage of the hypertensive patients also favors the idea of a renal humoral mechanism in which the effective vasoconstrictor substance is presumed to act directly on the musculature of the peripheral arterioles, and not by way of the vasomotor nerves. This is in keeping with the conclusions of Prinzmetal and Wilson (337) and of Pickering (320). The frequent fall of blood pressure in the late stage of pregnancy in animals with experimental renal hypertension (58, 77, 86, 87, 111, 292) remains as unexplained as does a similar fall which has been observed by many obstetricians in some hypertensive pregnant women. The observation of polydypsia and polyuria, in rats with experimental renal hypertension (289), has not been emphasized in human hypertension and has not been observed in hypertensive dogs, but the diuretic effect of renin injected intravenously into animals has been mentioned (272). This should be investigated further.

Renal blood flow is reduced in most cases of human hypertension (135, 154 384) and in experimental renal hypertension (306) in animals. The indirect studies (135, 154, 384) of blood flow through the kidney in man do not demonstrate clearly the primary effect of the sclerosis of the afferent arterioles, because the vasospasm of efferent arterioles which results in high glomerular filtration fraction tends to mask it. Interestingly enough, although the interference with afferent flow is definite, in the animals, and obviously brought about by constriction of the main renal artery, yet the same indirect signs of efferent vasospasm and high glomerular filtration fraction occur in the hypertensive animals. In both, the latter effects may be due to the humoral mechanism of renal origin. In both man and animals the hypertension may or may not be accompanied by disturbance of renal excretory function. An increase in the concentration of guanidine in the blood of animals (49, 51) and man (260, 261), in the malignant phase of hypertension, has been demonstrated; but it has little or no significance with relation to the hypertension, because it occurs also in bilaterally nephrectomized animals that have the azotemia but not the hypertension (80). The presence of renin has been demonstrated in the renal venous blood of ischemic kidneys of man and animals. Although renin has been demonstrated in the systemic blood of recently hypertensive dogs (215), and man with hypertension due to acute glomerulonephritis, yet the failure to demonstrate it in the systemic blood of dogs with long standing renal hypertension and patients with benign essential hypertension may be only because the amount of blood used for the tests has been inadequate and the methods have lacked sensitivity. Whether the humoral mechanism is effective only in the relatively acute stages of hypertension, or whether in the later stages, as has been suggested (39, 285), there is a greatly increased sensitivity to hypertensin, remains to be determined. These matters deserve much more study. In the benign phase of hypertension, in both man and animals, cardiac hypertrophy develops, affecting mainly the left ventricle, and medial hypertrophy of the arterial vessels also occurs in both (252). In the malignant phase, in both, there are the identical typical vascular lesions, arteriolar necrosis, fibrinoid degeneration and necrotizing arteriolitis (147). As in human, so also in experimental renal hypertension, the level of blood pressure tends to go to a higher level in hypertensive animals which gain weight (43), but a high protein diet or the ingestion of a large amount of urea has not been found to increase the blood pressure of hypertensive dogs (183). Although the elevated systolic blood pressure of hyperthyroidism is relieved by thyroidectomy, yet it is doubtful that any of the known endocrine organs plays a primary part in either essential hypertension associated with vascular disease, in man, or in experimental renal hypertension in animals. There are definite indications that the adrenal cortical hormones may play a secondary part in the development and maintenance of experimental renal and human essential hypertension (85, 125, 128, 318, 319, 342).

It would be remarkable indeed if, despite all these close similarities between human essential hypertension, associated with vascular disease, and a type of experimental hypertension that is obviously of renal origin, the former would

not prove to be of renal origin. In the event that the renal origin of this form of human hypertension should become established, it would still be necessary to determine the cause of the arterial and arteriolar sclerosis which, when it affects the kidneys to a sufficient degree, initiates the humoral mechanism of the hypertension. It is still a fascinating subject for investigation and much remains to be done.*

* Recently there was published a translation, in English, by Dexter, of the book on Renal Hypertension by Braun-Menendez and collaborators (37a). In the prologue, Houssay has written, "In the clinic, the existence of hypertension from renal ischemia, similar to experimental renal hypertension has not only been demonstrated, but the hypertension has been cured by removal of the diseased kidney in some cases of unilateral renal disease.

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